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(54) Title: ELECTRONIC METHODS FOR THE DETECTION OF ANALYTES

(57) Abstract

The present invention is directed to the detection of target analytes using electronic techniques, particularly AC techniques.

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ELECTRONIC METHODS FOR THE
DETECTION OF ANALYTES

CROSS-REFERENCED TO RELATED APPLICATIONS

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This is a continuing application of Application Serial No. 60/049,489, filed 12 June 1997.

FIELD OF THE INVENTION

10   The invention relates to analytical methods and apparatus, and particularly to the detection of analytes, including biomolecules, using electronic techniques, particularly AC techniques.

BACKGROUND OF THE INVENTION

15   There are a number of assays and sensors for the detection of the presence and/or concentration of specific substances in fluids and gases. Many of these rely on specific ligand/antiligand reactions as the mechanism of detection. That is, pairs of substances (i.e. the binding pairs or ligand/antiligands) are known to bind to each other, while binding little or not at all to other substances. This has been the focus of a number of techniques that utilize these binding pairs for the detection of the complexes.
20   These generally are done by labeling one component of the complex in some way, so as to make the entire complex detectable, using, for example, radioisotopes, fluorescent and other optically active molecules, enzymes, etc.

Other assays rely on electronic signals for detection. Of particular interest are biosensors. At least two
25   types of biosensors are known; enzyme-based or metabolic biosensors and binding or bioaffinity sensors. See for example U.S. Patent No. 4,713,347; 5,192,507; 4,920,047; 3,873,267; and references disclosed therein. While some of these known sensors use alternating current (AC) techniques, these techniques are generally limited to the detection of differences in bulk (or dielectric) impedance, and rely on the use of mediators in solution to shuttle the charge to the electrode.
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Recently, there have been several preliminary reports on the use of very short connections between a binding ligand and the electrode, for direct detection, i.e. without the use of mediators. See Lötzbeyer et al., Bioelectrochemistry and Bioenergetics 42:1-6 (1997); Dong et al., Bioelectrochemistry and Bioenergetics 42:7-13 (1997).

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In addition, there are a number of reports of self-assembled monolayers of conjugated oligomers on surfaces such as gold. See for example Cygan et al., J. Am. Chem. Soc. 120:2721 (1998).

5   In addition, Charych et al. report on the direct colormetric detection of a receptor-ligand interaction using a bilayer assembly (Science 261:585 (1993).

Accordingly, it is an object of the invention to provide novel methods and compositions for the detection of target analytes using AC techniques.

10   SUMMARY OF THE INVENTION

Accordingly, in accordance with the above objects, the present invention provides methods of detecting a target analyte in a test sample comprising a redox active molecule and an analyte The method comprises applying an input signal to the test sample and detecting a change in the faradaic impedance

15 of the system as a result of the association of the redox active molecule with the analyte.

In an additional aspect, the invention provides methods binding the target analyte to a redox active complex comprising a redox active molecule and a binding ligand which will bind the target analyte, followed by detection of a change in the faradaic impedance of the system as a result of the association

20 of the redox active molecule with the target analyte, if present.

The methods further comprising applying a first input signal to said redox active complex; the input signal can comprise an AC component and/or a DC component.

25 In a further aspect, the invention provides apparatus for the detection of analyte in a test sample, comprising a test chamber comprising at least a first and a second measuring electrode, wherein the first measuring electrode comprises a covalently attached ligand for an analyte, and an AC/DC voltage source electrically connected to the test chamber.

30 In an additional aspect, the present invention provides metal ion sensors comprising electrodes comprising self-assembled monolayers and at least one metal ion ligand or chelate covalently attached to the electrode via a conductive oligomer.

35   BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the synthetic scheme for a conductive polymer containing an aromatic group with a substitution group. The conductive oligomer is a phenyl-acetylene Structure 5 oligomer with a single

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methyl R group on each phenyl ring, although other oligomers may be used, and terminates in an ethyl pyridine protecting group, as described herein, for attachment to gold electrodes.

Figure 2 depicts the synthetic scheme for making a redox active complex comprising a RAM, in this
5　case ferrocene, to a protein binding ligand, in this case an antibody, using a standard coupling reaction. As will be appreciated by those in the art, any proteins that either contain suitable amines or can be derivatized to contain a suitable amine can be added in this manner. Alternatively, the amine may be added to the RAM and the BL contains the carboxylic acid. Similarly, while this figure depicts the attachment of a RAM (ferrocene), a similar reaction may be done with a conductive oligomer
10　terminating in a carboxylic acid (or an amine), for attachment to a proteinaceous binding ligand. In addition, while not depicted, "Z" linkers, as described below, may be added in between the components.

Figure 3 depicts the synthetic scheme of a System 3 type sensor, comprising a conductive oligomer containing a redox active complex of a RAM (in this case ferrocene), with a binding ligand (in this case a
15　biotin derivative). Any number of other RAMs and BLs may be used. Additional "Z" linkers, as described below, may be added in between the components. As for figure 2, a standard coupling agent (carbodimide) is used, which allows the attachment of virtually any amine- or carboxylic acid-containing moieties. The first subunit of the conductive oligomer is used, and then subsequent subunits are added.

20　Figure 4 depicts a sensor of the invention, directed to the detection of antibodies to a drug. Upon the introduction of a patient sample, with binding of the antibody to its antigen, the environment of the RAM is altered, leading to a detectable change in the signal (i.e. an alteration in the faradaic impedance). The redox potential of the RAM may be altered, or there may be a signal increase or alteration. As will be appreciated by those in the art, the drug in this case could be replaced by virtually any binding
25　ligand. In addition, this type of reaction may be run as a standard competitive type assay.

Figures 5A, 5B and 5C depict some possible ion sensors. The binding of ions such as Li+, Mg+2 or Na+ can alter the redox potential of the ferrocene by altering the electron withdrawing properties of the crown ethers, thus effecting a change in the signal upon binding.
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Figures 6A, 6B, 6C, 6D and 6E depict a metal ion sensor embodiment of the invention. Figure 6A depicts a chelate metal ion binding ligand, in this case phenanthroline, that was subsequently attached to a gold electrode, with a monolayer present. Figures 6B and 6C depict AC scans in the absence (6B) and presence (6C) of $FeCl_2$, showing a peak around 450 mV, the redox potential of the iron. Figure 6D
35　depicts the same composition in the presence of $Ru(NH_3)_4PyCl$, with a peak at around 650 mV. Figure 6E depicts the same composition in the presence of $K_4Fe^{II}(CN)_6$, also with a peak around 650 mV.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the detection of analytes using alternating current (AC) (also sometimes referred to as alternating voltage (AV)) techniques. The invention is based on the fact that

5   at least one redox property of a redox active molecule may be altered as a result of its association with a target analyte. Without being bound by theory, it appears that changes in the environment of the redox active molecule can result in altered redox properties. That is, upon association of the analyte and the redox active molecule in some way, a measurable redox property of the redox active molecule changes, thus allowing the detection of the analyte. In particular, it has been discovered that relatively

10   small changes in measurable redox properties can be detected using AC techniques, enabling a variety of possible biosensors.

The change in a redox property of the redox active molecule is a result of the association with an analyte. This may be due to a binding event, which may alter the conformation or accessibility of the

15   redox active molecule, and/or a change in the local environment of the redox active molecule (for example in the solvent reorganization energy), both of which will alter the faradaic impedance of the system, which in turn results in a characteristic output signal, i.e. a different output signal than is received in the absence of the target analyte.

20   Accordingly, the present invention is directed to the detection of analytes using changes in the faradaic impedance of the system as a result of the binding or association of an analyte. By "faradaic impedance" herein is meant the impedance between the redox active molecule and the electrode. Changes in capacitance (e.g. due to binding of compounds to the surface or bulk dielectric capacitance) are not included in the definition of changes in faradaic impedance. This is quite different

25   from the bulk or dielectric impedance, which is the impedance of the bulk solution between the electrodes. Many factors may change the faradaic impedance which may not effect the bulk impedance, and vice versa. As described herein, any number of perturbations of the system can result in an altered faradaic impedance, which may then serve as the basis of an assay. These include, but are not limited to, changes in electronic coupling of the redox active molecule and the electrode (often

30   referred to as H_{AB} in the literature); changes in λ, the nuclear reorganization energy, which is usually dominated by the solvent reorganization energy; changes in E_0 of the redox active molecule; the charge transfer impedance of the redox active molecule in the system; the mass transfer impedance of the redox active molecule in the system; changes in the redox active molecule, including exchange of ligands or metal ions; etc.

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Systems relying on changes in faradaic impedance can be distinguished from prior art systems on the basis of the use of mediators. That is, prior art systems usually rely on the use of soluble mediators to shuttle electrons between the redox active molecules and the electrode; however, the present invention relies on direct electron transfer between the redox active molecule and the electrode, generally

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through the use of conductive oligomers. Thus, the methods of the present invention are generally run in the absence of soluble mediators that serve to electronically mediate the redox active molecule and the electrode. That is, the redox active molecules (RAMs) are directly attached to the electrodes of the invention, rather than relying on bulk diffusion mechanisms. Mediators in this context are to be
5   distinguished by co-reductants and co-oxidants, as generally described below.

Generally, compositions and methods described in PCT US97/20014, hereby explicitly incorporated herein by reference in its entirety, find use in the present invention.

10   Thus the present invention is directed to methods and compositions for the detection of target analytes in test samples. By "target analyte" or "analyte" or grammatical equivalents herein is meant any molecule, compound or particle to be detected. As outlined below, target analytes preferably bind to binding ligands, as is more fully described below. As will be appreciated by those in the art, a large number of analytes may be detected using the present methods; basically, any target analyte for which
15   a binding ligand, described below, may be made may be detected using the methods of the invention.

Suitable analytes include organic and inorganic molecules, including biomolecules. In a preferred embodiment, the analyte may be an environmental pollutant (including pesticides, insecticides, toxins, etc.); a chemical (including solvents, polymers, organic materials, etc.); therapeutic molecules
20   (including therapeutic and abused drugs, antibiotics, etc.); biomolecules (including hormones, cytokines, proteins, lipids, carbohydrates, cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, etc); whole cells (including procaryotic (such as pathogenic bacteria) and eukaryotic cells, including mammalian tumor cells); viruses (including retroviruses, herpesviruses, adenoviruses, lentiviruses, etc.); and spores; etc. Particularly preferred
25   analytes are environmental pollutants; nucleic acids; proteins (including enzymes, antibodies, antigens, growth factors, cytokines, etc); therapeutic and abused drugs; cells; and viruses.

In a preferred embodiment, the target analytes are not nucleic acids. Similarly, a preferred embodiment utilizes target analytes that are not glucose, and redox active complexes that do not
30   contain glucose oxidase.

In a preferred embodiment, the target analyte is a protein. As will be appreciated by those in the art, there are a large number of possible proteinaceous target analytes that may be detected using the present invention. By "proteins" or grammatical equivalents herein is meant proteins, oligopeptides and
35   peptides, derivatives and analogs, including proteins containing non-naturally occurring amino acids and amino acid analogs, and peptidomimetic structures. The side chains may be in either the (R) or the (S) configuration. In a preferred embodiment, the amino acids are in the (S) or L-configuration. As discussed below, when the protein is used as a binding ligand, it may be desirable to utilize protein analogs to retard degradation by sample contaminants.

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Suitable protein target analytes include, but are not limited to, (1) immunoglobulins, particularly IgEs, IgGs and IgMs, and particularly therapeutically or diagnostically relevant antibodies, including but not limited to, for example, antibodies to human albumin, apolipoproteins (including apolipoprotein E), human chorionic gonadotropin, cortisol, α-fetoprotein, thyroxin, thyroid stimulating hormone (TSH),

5   antithrombin, antibodies to pharmaceuticals (including antieptileptic drugs (phenytoin, primidone, carbariezepin, ethosuximide, valproic acid, and phenobarbitol), cardioactive drugs (digoxin, lidocaine, procainamide, and disopyramide), bronchodilators (theophylline), antibiotics (chloramphenicol, sulfonamides), antidepressants, immunosuppresants, abused drugs (amphetamine, methamphetamine, cannabinoids, cocaine and opiates) and antibodies to any number of viruses

10 (including orthomyxoviruses, (e.g. influenza virus), paramyxoviruses (e.g respiratory syncytial virus, mumps virus, measles virus), adenoviruses, rhinoviruses, coronaviruses, reoviruses, togaviruses (e.g. rubella virus), parvoviruses, poxviruses (e.g. variola virus, vaccinia virus), enteroviruses (e.g. poliovirus, coxsackievirus), hepatitis viruses (including A, B and C), herpesviruses (e.g. Herpes simplex virus, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus), rotaviruses, Norwalk viruses, hantavirus,

15 arenavirus, rhabdovirus (e.g. rabies virus), retroviruses (including HIV, HTLV-I and -II), papovaviruses (e.g. papillomavirus), polyomaviruses, and picornaviruses, and the like), and bacteria (including a wide variety of pathogenic and non-pathogenic prokaryotes of interest including Bacillus; Vibrio, e.g. *V. cholerae;* Escherichia, e.g. Enterotoxigenic *E. coli,* Shigella, e.g. *S. dysenteriae;* Salmonella, e.g. *S. typhi;* Mycobacterium e.g. *M. tuberculosis, M. leprae;* Clostridium, e.g. *C. botulinum, C. tetani, C.*

20 *difficile, C.perfringens;* Cornyebacterium, e.g. *C. diphtheriae;* Streptococcus, *S. pyogenes, S. pneumoniae;* Staphylococcus, *e.g. S. aureus;* Haemophilus, e.g. *H. influenzae;* Neisseria, e.g. *N. meningitidis, N. gonorrhoeae;* Yersinia, e.g. *G. lambliaY. pestis,* Pseudomonas, e.g. *P. aeruginosa, P. putida;* Chlamydia, e.g. *C. trachomatis;* Bordetella, e.g. *B. pertussis;* Treponema, e.g. *T. palladium;* and the like); (2) enzymes (and other proteins), including but not limited to, enzymes used as indicators of or

25 treatment for heart disease, including creatine kinase, lactate dehydrogenase, aspartate amino transferase, troponin T, myoglobin, fibrinogen, cholesterol, triglycerides, thrombin, tissue plasminogen activator (tPA); pancreatic disease indicators including amylase, lipase, chymotrypsin and trypsin; liver function enzymes and proteins including cholinesterase, bilirubin, and alkaline phosphotase; aldolase, prostatic acid phosphatase, terminal deoxynucleotidyl transferase, and bacterial and viral enzymes

30 such as HIV protease; (3) hormones and cytokines (many of which serve as ligands for cellular receptors) such as erythropoietin (EPO), thrombopoietin (TPO), the interleukins (including IL-1 through IL-17), insulin, insulin-like growth factors (including IGF-1 and -2), epidermal growth factor (EGF), transforming growth factors (including TGF-α and TGF-β), human growth hormone, transferrin, epidermal growth factor (EGF), low density lipoprotein, high density lipoprotein, leptin, VEGF, PDGF,

35 ciliary neurotrophic factor, prolactin, adrenocorticotropic hormone (ACTH), calcitonin, human chorionic gonadotropin, cotrisol, estradiol, follicle stimulating hormone (FSH), thyroid-stimulating hormone (TSH), leutinzing hormone (LH), progeterone, testosterone, ; and (4) other proteins (including α-fetoprotein, carcinoembryonic antigen CEA.

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In addition, any of the biomolecules for which antibodies may be detected may be detected directly as well; that is, detection of virus or bacterial cells, therapeutic and abused drugs, etc., may be done directly.

5   Suitable target analytes include carbohydrates, including but not limited to, markers for breast cancer (CA15-3, CA 549, CA 27.29), mucin-like carcinoma associated antigen (MCA), ovarian cancer (CA125), pancreatic cancer (DE-PAN-2), and colorectal and pancreatic cancer (CA 19, CA 50, CA242).

Suitable target analytes include metal ions, particularly heavy and/or toxic metals, including but not
10   limited to, aluminum, arsenic, cadmium, selenium, cobalt, copper, chromium, lead, silver and nickel.

In a preferred embodiment, the target analyte is added to or introduced to a redox active molecule or redox active complex. By "redox active molecule" or "RAM" or "electron transfer moiety" or "ETM" herein is meant a compound which is capable of reversibly, semi-reversibly, or irreversibly transferring
15   one or more electrons. The terms "electron donor moiety", "electron acceptor moiety", and "electron transfer moieties" or grammatical equivalents herein refers to molecules capable of electron transfer under certain conditions. It is to be understood that electron donor and acceptor capabilities are relative; that is, a molecule which can lose an electron under certain experimental conditions will be able to accept an electron under different experimental conditions. It is to be understood that the
20   number of possible electron donor moieties and electron acceptor moieties is very large, and that one skilled in the art of electron transfer compounds will be able to utilize a number of compounds in the present invention. Preferred electron transfer moieties include, but are not limited to, transition metal complexes, organic electron transfer moieties, and electrodes.

25   In a preferred embodiment, the electron transfer moieties are transition metal complexes. Transition metals include those whose atoms have a partial or complete d shell of electrons; elements having the atomic numbers 21-30, 39-48, 57-80 and the lanthanide series. Suitable transition metals for use in the invention include, but are not limited to, cadmium (Cd), copper (Cu), cobalt (Co), palladium (Pd), zinc (Zn), iron (Fe), ruthenium (Ru), rhodium (Rh), osmium (Os), rhenium (Re), platinium (Pt), scandium
30   (Sc), titanium (Ti), Vanadium (V), chromium (Cr), manganese (Mn), nickel (Ni), Molybdenum (Mo), technetium (Tc), tungsten (W), and iridium (Ir). That is, the first series of transition metals, the platinum metals (Ru, Rh, Pd, Os, Ir and Pt), along with Fe, Re, W, Mo and Tc, are preferred. Particularly preferred are ruthenium, rhenium, osmium, platinium, cobalt and iron.

35   The transition metals are complexed with a variety of ligands, generally depicted herein as "L", to form suitable transition metal complexes, as is well known in the art. Suitable ligands fall into two categories: ligands which use nitrogen, oxygen, sulfur, carbon or phosphorus atoms (depending on the metal ion) as the coordination atoms (generally referred to in the literature as sigma (σ) donors) and organometallic ligands such as metallocene ligands (generally referred to in the literature as pi (π)

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donors, and depicted herein as L_m). Suitable nitrogen donating ligands are well known in the art and include, but are not limited to, NH_2; NHR; NRR'; pyridine; pyrazine; isonicotinamide; imidazole; bipyridine and substituted derivatives of bipyridine; terpyridine and substituted derivatives; phenanthrolines, particularly 1,10-phenanthroline (abbreviated phen) and substituted derivatives of

5   phenanthrolines such as 4,7-dimethylphenanthroline and dipyridol[3,2-a:2',3'-c]phenazine (abbreviated dppz); dipyridophenazine; 1,4,5,8,9,12-hexaazatriphenylene (abbreviated hat); 9,10-phenanthrenequinone diimine (abbreviated phi); 1,4,5,8-tetraazaphenanthrene (abbreviated tap); 1,4,8,11-tetra-azacyclotetradecane (abbreviated cyclam) and isocyanide. Substituted derivatives, including fused derivatives, may also be used. In some embodiments, porphyrins and substituted

10   derivatives of the porphyrin family may be used. See for example, Comprehensive Coordination Chemistry, Ed. Wilkinson et al., Pergammon Press, 1987, Chapters 13.2 (pp73-98), 21.1 (pp. 813-898) and 21.3 (pp 915-957), all of which are hereby expressly incorporated by reference.

Suitable sigma donating ligands using carbon, oxygen, sulfur and phosphorus are known in the art. For

15   example, suitable sigma carbon donors are found in Cotton and Wilkenson, Advanced Organic Chemistry, 5th Edition, John Wiley & Sons, 1988, hereby incorporated by reference; see page 38, for example. Similarly, suitable oxygen ligands include crown ethers, water and others known in the art. Phosphines and substituted phosphines are also suitable; see page 38 of Cotton and Wilkenson.

20   The oxygen, sulfur, phosphorus and nitrogen-donating ligands are attached in such a manner as to allow the heteroatoms to serve as coordination atoms.

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In a preferred embodiment, organometallic ligands are used. In addition to purely organic compounds for use as redox moieties, and various transition metal coordination complexes with δ-bonded organic

25   ligand with donor atoms as heterocyclic or exocyclic substituents, there is available a wide variety of transition metal organometallic compounds with π-bonded organic ligands (see Advanced Inorganic Chemistry, 5th Ed., Cotton & Wilkinson, John Wiley & Sons, 1988, chapter 26; Organometallics, A Concise Introduction, Elschenbroich et al., 2nd Ed., 1992, VCH; and Comprehensive Organometallic Chemistry II, A Review of the Literature 1982-1994, Abel et al. Ed., Vol. 7, chapters 7, 8, 10 & 11,

30   Pergamon Press, hereby expressly incorporated by reference). Such organometallic ligands include cyclic aromatic compounds such as the cyclopentadienide ion $[C_5H_5(-1)]$ and various ring substituted and ring fused derivatives, such as the indenylide (-1) ion, that yield a class of bis(cyclopentadieyl)metal compounds, (i.e. the metallocenes); see for example Robins et al., J. Am. Chem. Soc. 104:1882-1893 (1982); and Gassman et al., J. Am. Chem. Soc. 108:4228-4229 (1986), incorporated by

35   reference. Of these, ferrocene $[(C_5H_5)_2Fe]$ and its derivatives are prototypical examples which have been used in a wide variety of chemical (Connelly et al., Chem. Rev. 96:877-910 (1996), incorporated by reference) and electrochemical (Geiger et al., Advances in Organometallic Chemistry 23:1-93; and Geiger et al., Advances in Organometallic Chemistry 24:87, incorporated by reference) electron transfer or "redox" reactions. Metallocene derivatives of a variety of the first, second and third row

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transition metals are potential candidates as redox moieties. Other potentially suitable organometallic ligands include cyclic arenes such as benzene, to yield bis(arene)metal compounds and their ring substituted and ring fused derivatives, of which bis(benzene)chromium is a prototypical example, Other acyclic π-bonded ligands such as the allyl(-1) ion, or butadiene yield potentially suitable organometallic

5   compounds, and all such ligands, in conjuction with other π-bonded and δ-bonded ligands constitute the general class of organometallic compounds in which there is a metal to carbon bond. Electrochemical studies of various dimers and oligomers of such compounds with bridging organic ligands, and additional non-bridging ligands, as well as with and without metal-metal bonds are potential candidate redox moieties.

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As described herein, any combination of ligands may be used. Preferred combinations include: a) all ligands are nitrogen donating ligands; b) all ligands are organometallic ligands; and c) the ligand at the terminus of the conductive oligomer is a metallocene ligand and the ligand provided by the binding ligand is a nitrogen donating ligand, with the other ligands, if needed, are either nitrogen donating

15   ligands or metallocene ligands, or a mixture.

In addition, it may be desirable to use coordination sites of the transition metal ion for attachment of the redox active molecule to either a binding ligand (directly or indirectly using a linker), to form a redox active complex, or to the electrode (frequently using a spacer such as a conductive oligomer, as is

20   more fully described below), or both. Thus for example, when the redox active molecule is directly joined to a binding ligand, one, two or more of the coordination sites of the metal ion may be occupied by coordination atoms supplied by the binding ligand (or by the linker, if indirectly joined). In addition, or alternatively, one or more of the coordination sites of the metal ion may be occupied by a spacer used to attach the redox active molecule to the electrode.

25

In addition to transition metal complexes, other organic electron donors and acceptors may be used in the invention. These organic molecules include, but are not limited to, riboflavin, xanthene dyes, azine dyes, acridine orange, N,N'-dimethyl-2,7-diazapyrenium dichloride (DAP^{2+}), methylviologen, ethidium bromide, quinones such as N,N'-dimethylanthra(2,1,9-def:6,5,10-d'e'f')diisoquinoline dichloride

30   (ADIQ^{2+}); porphyrins ([meso-tetrakis(N-methyl-x-pyridinium)porphyrin tetrachloride], varlamine blue B hydrochloride, Bindschedler's green; 2,6-dichloroindophenol, 2,6-dibromophenolindophenol; Brilliant crest blue (3-amino-9-dimethyl-amino-10-methylphenoxyazine chloride), methylene blue; Nile blue A (aminoaphthodiethylaminophenoxazine sulfate), indigo-5,5',7,7'-tetrasulfonic acid, indigo-5,5',7-trisulfonic acid; phenosafranine, indigo-5-monosulfonic acid; safranine T; bis(dimethylglyoximato)-

35   iron(II) chloride; induline scarlet, neutral red, anthracene, coronene, pyrene, 9-phenylanthracene, rubrene, binaphthyl, DPA, phenothiazene, fluoranthene, phenanthrene, chrysene, 1,8-diphenyl-1,3,5,7-octatetracene, naphthalene, acenaphthalene, perylene, TMPD and analogs and subsitituted derivatives of these compounds.

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In one embodiment, the electron donors and acceptors are redox proteins as are known in the art. However, redox proteins in many embodiments are not preferred.

5   The choice of the specific electron transfer moieties will be influenced by the type of electron transfer detection used, as is generally outlined below.

In some embodiments, as is outlined below, the redox active molecule is actually the analyte to be detected; for example, when redox active proteins such as metalloenzymes, cytochrome c, etc. are to be detected, they may serve as the redox active molecule. Alternatively, some metal analytes,
10 particularly heavy metals, can also serve as the redox active molecule, in general with chelating ligands as is described herein; see for example Figure 6.

Generally, the target analyte binds to a redox active complex. By "redox active complex" herein is meant a complex comprising at least one redox active molecule and at least one binding ligand, which,
15 as more fully described below, may be associated in a number of different ways. In some cases, the binding ligand may also be a redox active molecule. By "binding ligand" or grammatical equivalents herein is meant a compound that is used to probe for the presence of the target analyte, and that will bind to the analyte.

20 As will be appreciated by those in the art, the composition of the binding ligand will depend on the composition of the target analyte. Binding ligands for a wide variety of analytes are known or can be readily found using known techniques. For example, when the analyte is a protein, the binding ligands include proteins (particularly including antibodies or fragments thereof (FAbs, etc.)) or small molecules. When the analyte is a metal ion, the binding ligand generally comprises traditional metal ion ligands or
25 chelators, which together form the redox active molecule. Preferred binding ligand proteins include peptides. For example, when the analyte is an enzyme, suitable binding ligands include substrates and inhibitors. Antigen-antibody pairs, receptor-ligands, and carbohydrates and their binding partners are also suitable analyte-binding ligand pairs. The binding ligand may be nucleic acid, when nucleic acid binding proteins are the targets; alternatively, as is generally described in U.S. Patents 5,270,163,
30 5,475,096, 5,567,588, 5,595,877, 5,637,459, 5,683,867,5,705,337, and related patents, hereby incorporated by reference, nucleic acid "aptomers" can be developed for binding to virtually any target analyte. Similarly, there is a wide body of literature relating to the development of binding partners based on combinatorial chemistry methods. In this embodiment, when the binding ligand is a nucleic acid, preferred compositions and techniques are outlined in PCT US97/20014, hereby incorporated by
35 reference.

By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have

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alternate backbones, comprising, for example, phosphoramide (Beaucage et al., Tetrahedron
49(10):1925 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al.,
Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem.
Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica

5   Scripta 26:141 91986)), phosphorothioate (Mag et al., Nucleic Acids Res. 19:1437 (1991); and U.S.
Patent No. 5,644,048), phosphorodithioate (Briu et al., J. Am. Chem. Soc. 111:2321 (1989), O-
methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach,
Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem.
Soc. 114:1895 (1992); Meier et al., Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566

10   (1993); Carlsson et al., Nature 380:207 (1996), all of which are incorporated by reference). Other
analog nucleic acids include those with positive backbones (Denpcy et al., Proc. Natl. Acad. Sci. USA
92:6097 (1995); non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141
and 4,469,863; Kiedrowshi et al., Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger et al., J. Am.
Chem. Soc. 110:4470 (1988); Letsinger et al., Nucleoside & Nucleotide 13:1597 (1994); Chapters 2

15   and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S.
Sanghui and P. Dan Cook; Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et
al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones,
including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC
Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P.

20   Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the
definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp169-176). Several nucleic
acid analogs are described in Rawls, C & E News June 2, 1997 page 35. All of these references are
hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone
may be done to facilitate the addition of RAMs or conductive oligomers, or to increase the stability and

25   half-life of such molecules in physiological environments.

As will be appreciated by those in the art, all of these nucleic acid analogs may find use in the present
invention. In addition, mixtures of naturally occurring nucleic acids and analogs can be made; for
example, at the site of conductive oligomer or RAM attachment, an analog structure may be used.

30   Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occuring nucleic acids
and analogs may be made.

In a preferred embodiment, the binding of the target analyte to the binding ligand is specific, and the
binding ligand is part of a binding pair. By "specifically bind" herein is meant that the ligand binds the

35   analyte, with specificity sufficient to differentiate between the analyte and other components or
contaminants of the test sample. However, as will be appreciated by those in the art, it will be possible
to detect analytes using binding which is not highly specific; for example, the systems may use different
binding ligands, for example an array of different ligands, and detection of any particular analyte is via
its "signature" of binding to a panel of binding ligands, similar to the manner in which "electronic noses"

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work. This finds particular utility in the detection of chemical analytes. The binding should be sufficient to remain bound under the conditions of the assay, including wash steps to remove non-specific binding. In some embodiments, for example in the detection of certain biomolecules, the disassociation constants of the analyte to the binding ligand will be less than about 10^{-4}-10^{-6} M^{-1}, with

5   less than about 10^{-5} to 10^{-9} M^{-1} being preferred and less than about 10^{-7} -10^{-9} M^{-1} being particularly preferred.

Together, when present, the redox active molecule and the binding ligand comprise a redox active complex. As mentioned above, in some cases the binding ligand is the redox active molecule, and thus

10   the redox active complex comprises the redox active binding ligand. Furthermore, in some embodiments, the target analyte is a redox active molecule, such as a metal ion or a metalloenzyme, etc.; in this case, a separate redox active molecule need not be used. In addition, there may be more than one binding ligand or redox active molecule per redox active complex, as is generally outlined below. The redox active complex may also contain additional moieties, such as cross-linking agents,

15   labels, etc., and linkers for attachment to the electrode. The addition (generally via non-covalent binding, although as outlined herein, some interactions may be considered covalent, or post-binding covalent attachment may occur, for example through the use of cross-linking agents) of the target analyte to the redox active complex forms an assay complex. By "assay complex" herein is meant the complex of components, including target analytes, binding ligands and redox active molecules, that

20   allows detection. The composition of the assay complex depends on the use of the different component outlined herein.

                                                                        t

In some embodiments, as is outlined below, the redox active complex is soluble. However, in a preferred embodiment, at least one component of the assay complex is covalently attached to an

25   electrode. In a preferred embodiment, it is generally a component of the redox active complex that is attached; that is, target analytes are not generally covalently attached to the electrode. That is, either the redox active molecule or the binding ligand is covalently attached to the electrode. By "electrode" herein is meant a conductive or semi-conductive composition, which, when connected to an electronic control and detection device, is able to transmit electrons to or from a RAM either in solution or on its

30   surface. Thus, an electrode is an electron transfer moiety as described herein. Preferred electrodes are known in the art and include, but are not limited to, certain metals and their oxides, including gold; platinum; palladium; silicon; aluminum; metal oxide electrodes including platinum oxide, titanium oxide, tin oxide, indium tin oxide, palladium oxide, silicon oxide, aluminum oxide, molybdenum oxide (Mo_2O_6), tungsten oxide (WO_3) and ruthenium oxides; and carbon (including glassy carbon electrodes, graphite

35   and carbon paste). Preferred electrodes include gold, silicon, carbon and metal oxide electrodes.

The electrodes described herein are depicted as a flat surface, which is only one of the possible conformations of the electrode and is for schematic purposes only. The conformation of the electrode will vary with the detection method used. For example, flat planar electrodes may be preferred for

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optical detection methods, or when arrays of binding ligands are made, thus requiring addressable locations for both synthesis and detection. Alternatively, for single probe analysis, the electrode may be in the form of a tube, with the conductive oligomers and binding ligands bound to the inner surface. This allows a maximum of surface area containing the target analytes to be exposed to a small volume of

5   sample.

The systems of the invention may take on any number of configurations, as outlined below.

In a preferred embodiment, the system is used to detect pollutants, such as organic pollutants, as is

10   depicted below in System 1:

System 1

	organic pollutant	
—F_1——X——F_2—TG		—F_1——X——F_2—TG
—F_1——X——F_2—RAM	→	—F_1——X——F_2—RAM
—F_1——X——F_2—TG		—F_1——X——F_2—TG
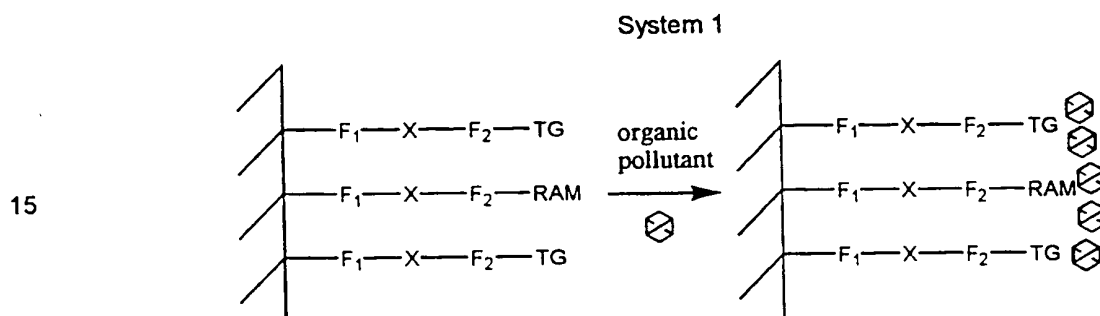
In System 1, as is described below, the hatched marks indicate an electrode, and there is preferably a

20   monolayer on the surface. F_1 is a linkage that allows the covalent attachment of the electrode and the conductive oligomer or insulator, including bonds, atoms or linkers such as is described herein, for example as "A", defined below. F_2 is a linkage that allows the covalent attachment of the conductive oligomer or insulator, and may be a bond, an atom or a linkage as is herein described. F_2 may be part of the conductive oligomer, part of the insulator, part of the terminal group, part of the redox active

25   complex or component, or exogenous to both, for example, as defined herein for "Z". X is a spacer (conductive oligomer, passivation agent or insulator, as required). RAM is a redox active molecule. TG is a terminal group, which may be chosen to influence the association of the target pollutant, such as an organic pollutant. Thus for example in this embodiment TG may be hydrophobic. The association of the pollutant on the surface will affect the local environment of the RAM, for example potentially by

30   changing the E_0 of the RAM or the solvent reorganization energy, and thus results in a change in the faradaic impedance of the system in the presence of the analyte. The association in this case is not specific for a particular analyte.

Systems 2, 3, 4 and 5 depict a similar situation except that a specific interaction is exploited. Thus, the

35   target analyte will bind to the binding ligand specifically, and is generally large as compared to the binding ligand and RAM. Upon binding, the local environment of the RAM is affected, for example potentially by changing the E_0 of the RAM or the solvent reorganization energy, and thus results in a change in the faradaic impedance of the system in the presence of the analyte. The target analyte in these cases could be protein, a cell, etc. In addition, any or all of these systems may be used with co-

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redoxants, as described below. Upon binding of the target analyte, the access of the co-redoxant to the RAM is restricted, thus resulting in either a different signal or a loss in signal, or both. In addition, as for all the systems depicted herein, the order or proximity of the individual molecules of the monolayer is not determinative.
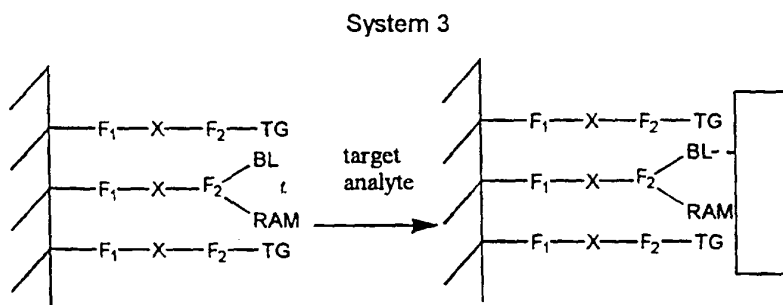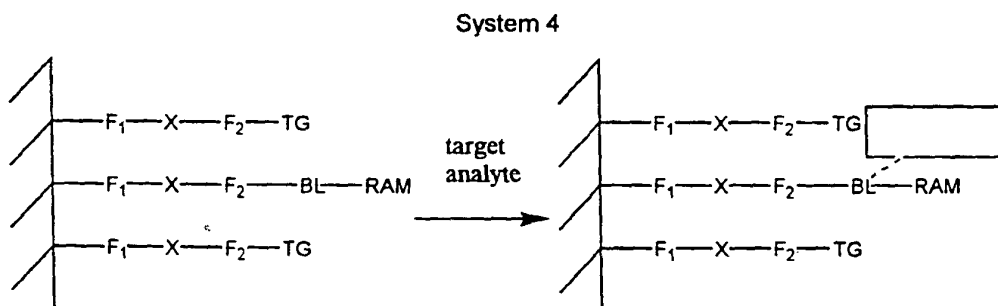
5
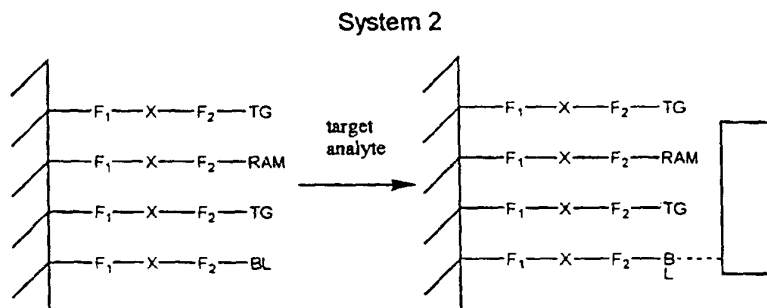
System 2

In System 2, there may be more than one RAM per binding ligand (BL); that is, the ratio of RAM to BL on the surface (depending on the relative size of the target analyte) may range from 1:1 to over 100:1. This allows an amplification of signal, in that more than one RAM is used to detect a single target analyte.

System 3

System 4

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System 5

5
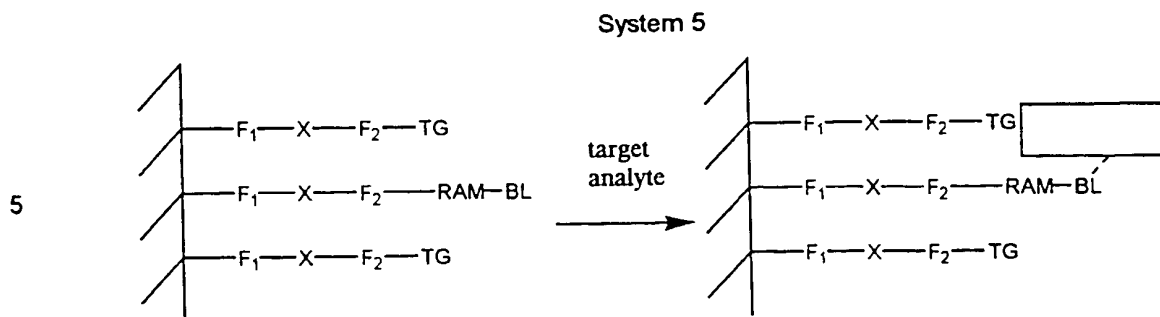
System 6 depicts a system in which binding of a target analyte theoretically affects the H_{AB} between the
10   RAM and the electrode:

System 6

15
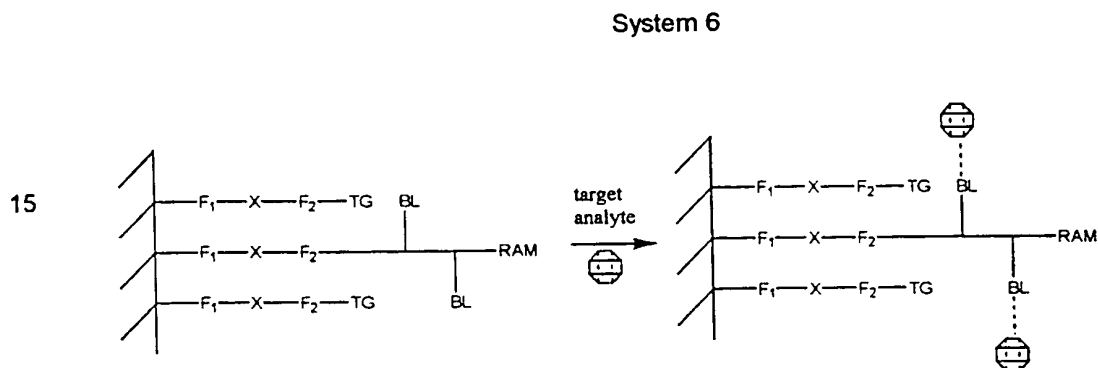
20   System 7 depicts a similar situation, except that the binding ligand is inherent in the attachment of the
RAM to the electrode; for example, it may be a peptide or nucleic acid to which the analyte binds:
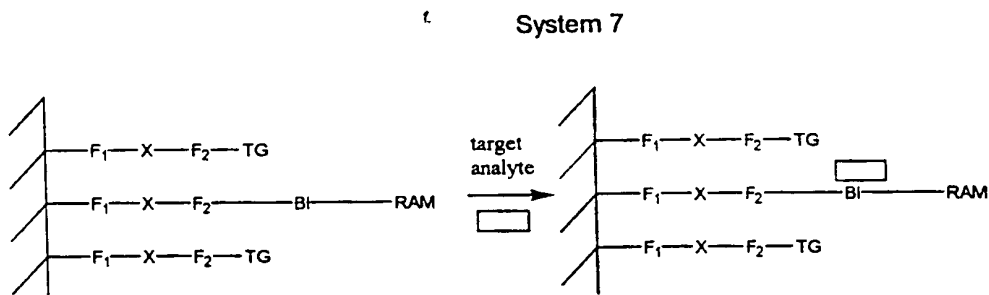
System 7

System 8 depicts a situation in which the analyte also serves as the redox active molecule; this is
particularly useful in the detection of metal ions, for example heavy metal ions, which are toxic. System
25   8 depicts a metal ion, M, and a metal ligand, ML, although as will be appreciated by those in the art, it is
quite possible to have the analyte in this case be a metalloprotein, with a BL, etc. As will be appreciated
by those in the art, System 8 is particularly useful in the detection of different metal ions, using an array
of different ligands; preferential binding of one metal over another would result in a panel of results that
can be correlated to metal ligand binding. Moreover, different metals may have different E_0s and thus
30   give different signals.

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System 8

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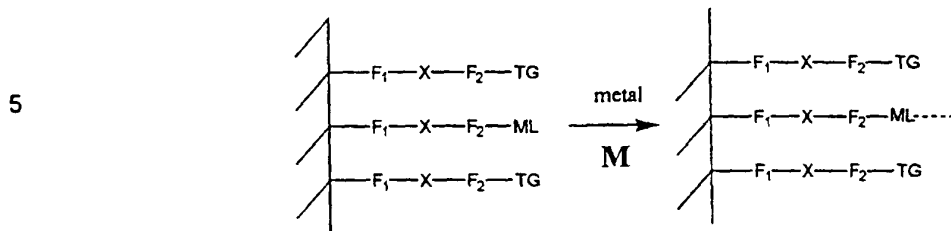
10   System 9 depicts a competitive-type assay which relies on a decrease in signal for detection. In this case, the target analyte is a ligand, for example carbon monoxide (CO), which are stronger ligands (SMLs, i.e. have higher binding constants) for a particular metal than the weaker metal ligand (WML) of the system.

System 9

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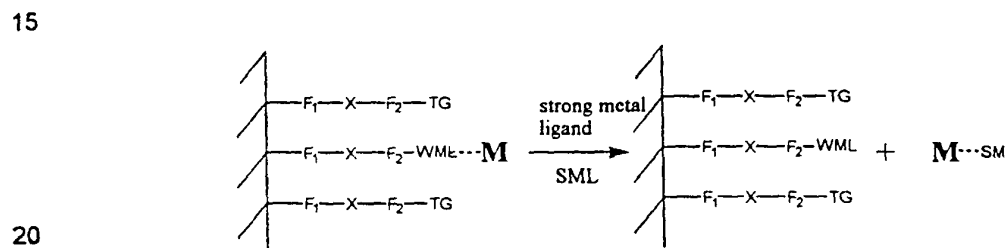
System 10 depicts a similar type of assay, which results in a change in signal rather than a decrease in signal. For example, E_0 and λ could both change as a result of a new ligand binding.

System 10

25

30
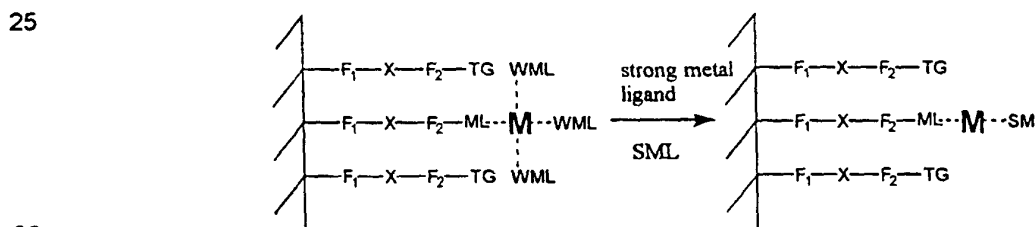
System 11 utilizes a change in the diffusion coefficient upon analyte binding for the change in faradaic impedance and mass transfer. In this embodiment, when the ligands are not covalently attached to an electrode, changes in the diffusion coefficient will alter the mass transfer impedance and thus the total

35   faradaic impedance. That is, in some circumstances the frequency response of a redox active complex will be limited by its diffusion coefficient. Also, the charge transfer impedance may be altered by the binding of an analyte. At high frequencies, a redox active complex may not diffuse rapidly enough to reversibly transfer its electron to the electrode at a rate sufficient to generate a strong output signal. At

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low frequencies, the molecule has sufficient time to diffuse, and thus an output signal can be detected. In this embodiment, the use of monolayers is generally not preferred.

5　Thus, the result of binding to form an assay complex will generally alter the diffusion coefficient of the redox active molecule. As a result, the faradaic impedance will change. This effect will be greatest when the binding partner is large in comparison to the redox active moiety; the redox active moiety will go from being relatively small, and thus diffusing quickly, to relatively large upon binding into a complex, and diffusing much more slowly; this results in the greatest changes and is thus preferred. Similarly, binding partners of roughly equal size can also result in a detectable signal.

10

Alternatively, it is also possible that binding of the redox active moiety to its binding partner will cause a decrease in size. For example, some protein structures, i.e. antibodies, may have "loose" conformations that are sterically bulky, that "tighten up" as a result of binding to its partner (i.e. an antigen).

15　　　　　　　　　　　　　　　System 11

20
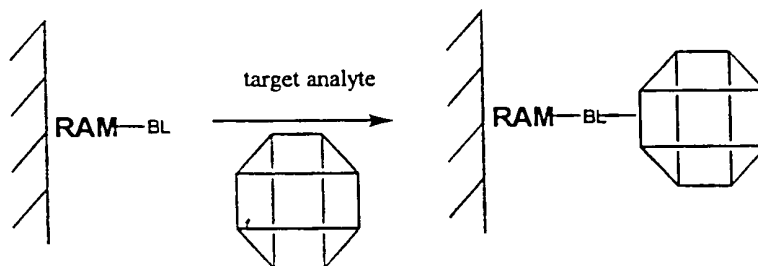
System 12 is similar to systems 10 and 11, as it is a sensor for different ligands, but it relies on a change
25　in ligands to result in a change in E_0 of the system. A similar system may be used with two metals; that is, instead of adding strong metal ligands, a different metal, with different affinity for the ligands may be added, resulting in a electrochemical change.
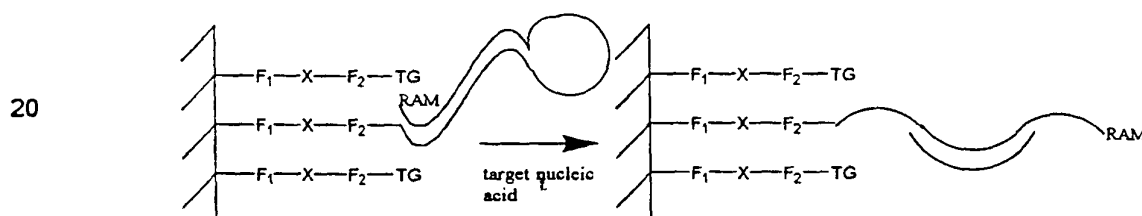
System 12

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35
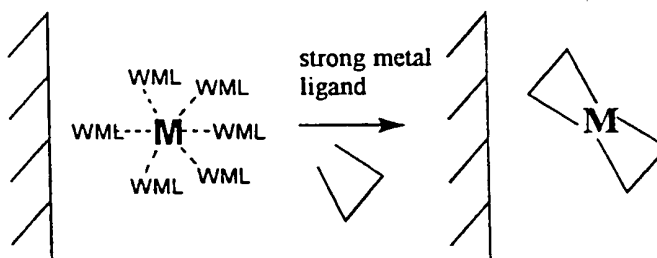
System 13 is a variation on previous systems, except that the RAM and the BL are closely associated or linked.

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System 13

5
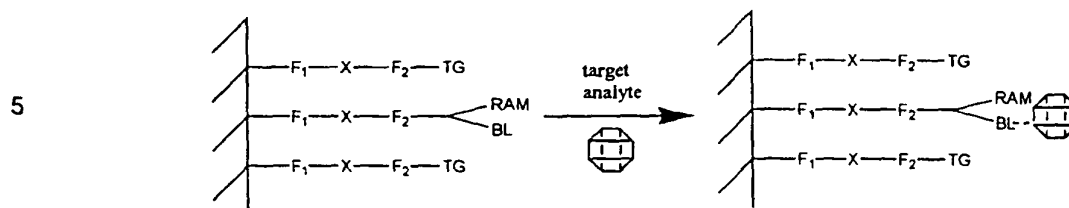
10   System 14 results in changes in faradaic impedance as a result of changes in E_0 or H_{AB}. In this case, the binding ligand will self-associate in some way, bringing the RAM into closer proximity to the electrode. For example, the binding ligand may be a nucleic acid (for example for the detection of a nucleic acid binding protein) or a protein (for example for the detection of proteins that inhibit or bind the binding ligand protein. Upon binding of the target, for example a protein, the conformation and thus the

15   local environment of the RAM changes, resulting in a detectable signal. System 15 could also be run in "reverse", wherein the association of the analyte brings the RAM into proximity of the surface.

System 14

20

25   System 15 uses two binding ligands, BL1 and BL2, which may be the same or different, to alter the environment of the RAM. It may be desirable to have one of the binding ligands be a somewhat "generic" binding ligand. Changes in E_0 and/or impedance will result in a detectable signal.
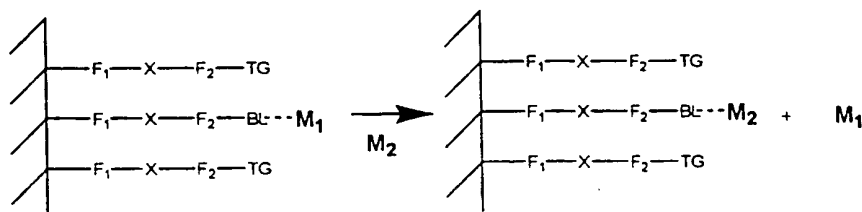
System 15

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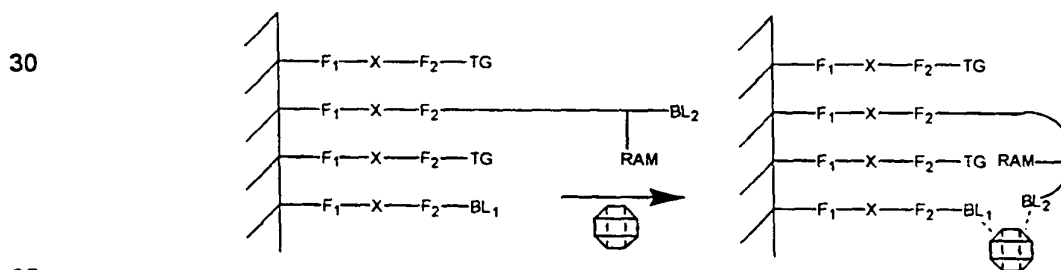
System 16 also relies on a decrease in signal. In this embodiment, a target analyte is used that will bind the metal ion-binding ligand complex in such a way as to render the metal unavailable to serve as a redox active molecule.

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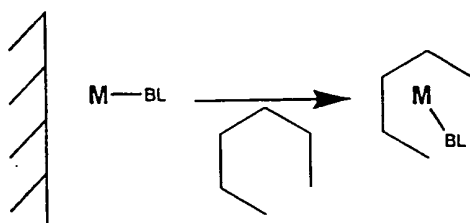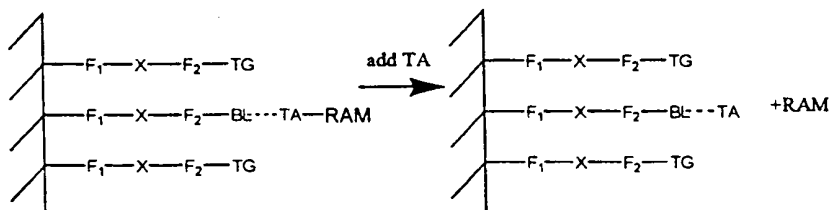
System 16

System 17 utilizes a change in metal ion affinity to a particular binding ligand to detect a change in the signal based on a different metal being present (resulting in a different E_0).
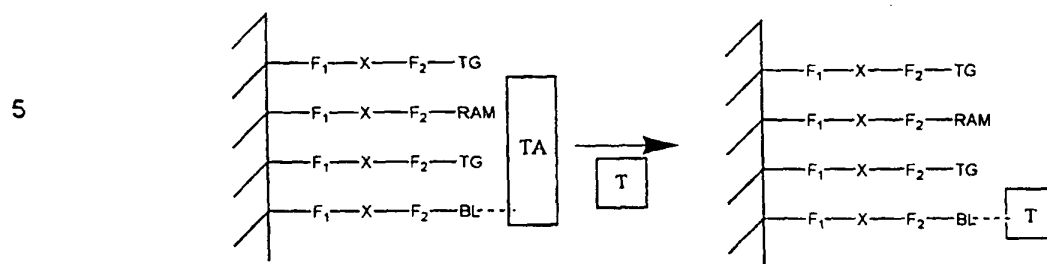
System 17

System 18 is similar to System 9 and depicts a competitive-type assay for detecting a target analyte. In System 15, a covalently attached target analyte or target analog (TA) is competed off of the binding ligand by the addition of the target analyte, resulting in a decrease in signal.
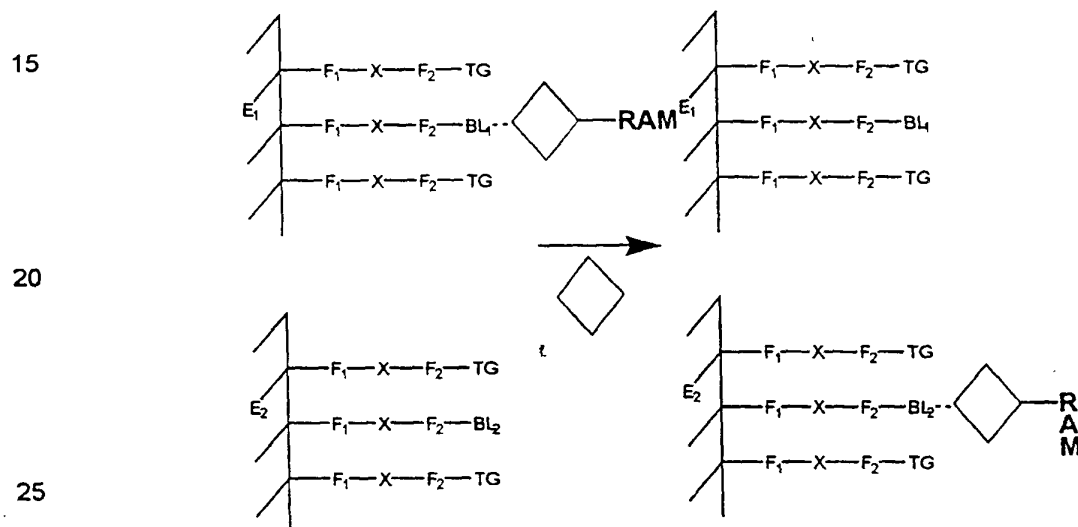
System 18

System 19 is a mixture of Systems 2 and 18, where the replacement of a bulky analog (TA) by a smaller target analyte (T) results in a different signal. For example, co-redoxant reactions could now occur. Alternatively, monolayers with "holes", that would allow current flow in the absence of the analog but do not in its presence, could also be used.

19

System 19

5

F_1—X—F_2—TG
F_1—X—F_2—RAM   TA   →   T
F_1—X—F_2—TG
F_1—X—F_2—BL---

F_1—X—F_2—TG
F_1—X—F_2—RAM
F_1—X—F_2—TG
F_1—X—F_2—BL--- T

10   System 20 depicts a two electrode system in a competitive-type assay. This is useful in that it allows detection of an increase in signal on the second electrode, which is generally preferable to the loss of a signal.

System 20

15

E_1
F_1—X—F_2—TG
F_1—X—F_2—BL---◇—RAM
F_1—X—F_2—TG

E_1
F_1—X—F_2—TG
F_1—X—F_2—BL
F_1—X—F_2—TG

20

↓

E_2
F_1—X—F_2—TG
F_1—X—F_2—BL$_2$
F_1—X—F_2—TG

E_2
F_1—X—F_2—TG
F_1—X—F_2—BL$_2$---◇—RAM
F_1—X—F_2—TG

25

    As will be appreciated by those in the art, System 20 may also be configured in several different ways. BL1 and BL2 may have different affinities for the same site on the target analyte or analog, or bind to different sites. Similarly, the other systems may also be run in two electrode systems.

30

    In addition, it is possible to use systems like those depicted above in several other embodiments. For example, since heat will change the faradaic impedance, the systems above could be used as a heat sensor. Similarly, attachment of the RAM to the electrode using a labile or cleavable bond can allow sensing of the cleaving agent based on a decrease in signal; for example, photolabile bonds can be

35   used to detect light (uv); substrates can be used to sense enzymes (proteases, nucleases, carbohydrases, lipases, etc.) or other cleaving agents, such as drugs that cut nucleic acids, etc.

    In the systems described above, the redox active complex is covalently attached to the electrode. This may be accomplished in any number of ways, as will be apparent to those in the art. In a preferred

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embodiment, one or both of the redox active molecule and the binding ligand are attached, via a spacer, to the electrode, using the techniques and compositions outlined below. By "spacer" herein is meant a moiety which holds the redox active complex off the surface of the electrode. In a preferred embodiment, the spacer used to attach the redox active molecule is a conductive oligomer as outlined

5   herein, although suitable spacer moieties include passivation agents and insulators as outlined below. The spacer moieties may be substantially non-conductive. In general, the length of the spacer is as outlined for conductive polymers and passivation agents. As will be appreciated by those in the art, if the spacer becomes too long, the electronic coupling between the redox active molecule and the electrode will decrease rapidly.

10

In a preferred embodiment, the redox active molecule will be attached via a conductive oligomer, such that detection of changes in faradaic impedance as between the redox active molecule and the electrode can be detected. Other components of the system may be attached using other spacers; for example, when the binding ligand and the redox active molecule are attached separately, as is

15   generally depicted in System 2, the binding ligand may be attached via a non-conductive oligomer spacer.

In a preferred embodiment, the spacer is a conductive oligomer. By "conductive oligomer" herein is meant a substantially conducting oligomer, preferably linear, some embodiments of which are referred

20   to in the literature as "molecular wires".

By "conductive oligomer" herein is meant a substantially conducting oligomer, preferably linear, some embodiments of which are referred to in the literature as "molecular wires". By "substantially conducting" herein is meant that the rate of electron transfer through the conductive oligomer is

25   generally not the rate limiting step in the detection of the target analyte, although as noted below, systems which use spacers that are the rate limiting step are also acceptable. Stated differently, the resistance of the conductive oligomer is less than that of the other components of the system. Generally, the conductive oligomer has substantially overlapping π-orbitals, i.e. conjugated π-orbitals, as between the monomeric units of the conductive oligomer, although the conductive oligomer may

30   also contain one or more sigma (σ) bonds. Additionally, a conductive oligomer may be defined functionally by its ability to pass electrons into or from an attached component. Furthermore, the conductive oligomer is more conductive than the insulators as defined herein.

In a preferred embodiment, the conductive oligomers have a conductivity, S, of from between about 10^{-6}

35   to about 10^4 $\Omega^{-1}cm^{-1}$, with from about 10^{-5} to about 10^3 $\Omega^{-1}cm^{-1}$ being preferred, with these S values being calculated for molecules ranging from about 20Å to about 200Å. As described below, insulators have a conductivity S of about 10^{-7} $\Omega^{-1}cm^{-1}$ or lower, with less than about 10^{-8} $\Omega^{-1}cm^{-1}$ being preferred. See generally Gardner et al., Sensors and Actuators A 51 (1995) 57-66, incorporated herein by reference.

21

Desired characteristics of a conductive oligomer include high conductivity, sufficient solubility in organic

solvents and/or water for synthesis and use of the compositions of the invention, and preferably

chemical resistance to reactions that occur i) during synthesis of the systems of the

invention, ii) during the attachment of the conductive oligomer to an electrode, or iii) during test assays.
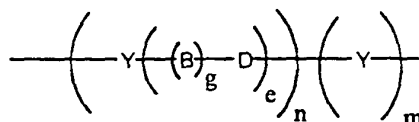
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The oligomers of the invention comprise at least two monomeric subunits, as described herein. As is

described more fully below, oligomers include homo- and hetero-oligomers, and include polymers.

In a preferred embodiment, the conductive oligomer has the structure depicted in Structure 1:

10   Structure 1

15   As will be understood by those in the art, all of the structures depicted herein may have additional

atoms or structures; i.e. the conductive oligomer of Structure 1 may be attached to redox active

molecules such as electrodes, transition metal complexes, organic electron transfer moieties, and

metallocenes, and to binding ligands, or to several of these. Unless otherwise noted, the conductive

oligomers depicted herein will be attached at the left side to an electrode; that is, as depicted in

20   Structure 1, the left "Y" is connected to the electrode as described herein and the right "Y", if present, is

attached to the redox active complex, either directly or through the use of a linker, as is described

herein.

In this embodiment, Y is an aromatic group, n is an integer from 1 to 50, g is either 1 or zero, e is an

25   integer from zero to 10, and m is zero or 1. When g is 1, B-D is a conjugated bond, preferably selected

from acetylene, alkene, substituted alkene, amide, azo, -C=N- (including -N=C-, -CR=N- and -N=CR-),

-Si=Si-, and -Si=C- (including -C=Si-, -Si=CR- and -CR=Si-). When g is zero, e is preferably 1, D is

preferably carbonyl, or a heteroatom moiety, wherein the heteroatom is selected from oxygen, sulfur,

nitrogen or phosphorus. Thus, suitable heteroatom moieties include, but are not limited to, -NH and -

30   NR, wherein R is as defined herein; substituted sulfur; sulfonyl (-SO$_2$-) sulfoxide (-SO-); phosphine oxide

(-PO- and -RPO-); and thiophosphine (-PS- and -RPS-). However, when the conductive oligomer is to

be attached to a gold electrode, as outlined below, sulfur derivatives are not preferred.

By "aromatic group" or grammatical equivalents herein is meant an aromatic monocyclic or polycyclic

35   hydrocarbon moiety generally containing 5 to 14 carbon atoms (although larger polycyclic rings

structures may be made) and any carbocylic ketone or thioketone derivative thereof, wherein the

carbon atom with the free valence is a member of an aromatic ring. Aromatic groups include arylene

groups and aromatic groups with more than two atoms removed. For the purposes of this application

aromatic includes heterocycle. "Heterocycle" or "heteroaryl" means an aromatic group wherein 1 to 5

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of the indicated carbon atoms are replaced by a heteroatom chosen from nitrogen, oxygen, sulfur, phosphorus, boron and silicon wherein the atom with the free valence is a member of an aromatic ring, and any heterocyclic ketone and thioketone derivative thereof. Thus, heterocycle includes thienyl, furyl, pyrrolyl, pyrimidinyl, oxalyl, indolyl, purinyl, quinolyl, isoquinolyl, thiazolyl, imidozyl, etc.

5

Importantly, the Y aromatic groups of the conductive oligomer may be different, i.e. the conductive oligomer may be a heterooligomer. That is, a conductive oligomer may comprise a oligomer of a single type of Y groups, or of multiple types of Y groups. Thus, in a preferred embodiment, when a barrier monolayer is used as is described below, one or more types of Y groups are used in the conductive

10   oligomer within the monolayer with a second type(s) of Y group used above the monolayer level. Thus, as is described herein, the conductive oligomer may comprise Y groups that have good packing efficiency within the monolayer at the electrode surface, and a second type(s) of Y groups with greater flexibility and hydrophilicity above the monolayer level to facilitate target analyte binding. For example, unsubstituted benzyl rings may comprise the Y rings for monolayer packing, and substituted benzyl

15   rings may be used above the monolayer. Alternatively, heterocylic rings, either substituted or unsubstituted, may be used above the monolayer. Additionally, in one embodiment, heterooligomers are used even when the conductive oligomer does not extend out of the monolayer.

The aromatic group may be substituted with a substitution group, generally depicted herein as R. R

20   groups may be added as necessary to affect the packing of the conductive oligomers, i.e. when the conductive oligomers form a monolayer on the electrode, R groups may be used to alter the association of the oligomers in the morfolayer. R groups may also be added to 1) alter the solubility of the oligomer or of compositions containing the oligomers; 2) alter the conjugation or electrochemical potential of the system; and 3) alter the charge or characteristics at the surface of the monolayer.

25

In a preferred embodiment, when the conductive oligomer is greater than three subunits, R groups are preferred to increase solubility when solution synthesis is done. However, the R groups, and their positions, are chosen to minimally effect the packing of the conductive oligomers on a surface, particularly within a monolayer, as described below. In general, only small R groups are used within the

30   monolayer, with larger R groups generally above the surface of the monolayer. Thus for example the attachment of methyl groups to the portion of the conductive oligomer within the monolayer to increase solubility is preferred, with attachment of longer alkoxy groups, for example, C3 to C10, is preferably done above the monolayer surface. In general, for the systems described herein, this generally means that attachment of sterically significant R groups is not done on any of the first three oligomer subunits,

35   depending on the length of the insulator molecules.

Suitable R groups include, but are not limited to, hydrogen, alkyl, alcohol, aromatic, amino, amido, nitro, ethers, esters, aldehydes, sulfonyl, silicon moieties, halogens, sulfur containing moieties, phosphorus containing moieties, and ethylene glycols. In the structures depicted herein, R is hydrogen when the

23

position is unsubstituted. It should be noted that some positions may allow two substitution groups, R and R', in which case the R and R' groups may be either the same or different.

By "alkyl group" or grammatical equivalents herein is meant a straight or branched chain alkyl group,
5   with straight chain alkyl groups being preferred. If branched, it may be branched at one or more positions, and unless specified, at any position. The alkyl group may range from about 1 to about 30 carbon atoms (C1 -C30), with a preferred embodiment utilizing from about 1 to about 20 carbon atoms (C1 -C20), with about C1 through about C12 to about C15 being preferred, and C1 to C5 being particularly preferred, although in some embodiments the alkyl group may be much larger. Also
10   included within the definition of an alkyl group are cycloalkyl groups such as C5 and C6 rings, and heterocyclic rings with nitrogen, oxygen, sulfur or phosphorus. Alkyl also includes heteroalkyl, with heteroatoms of sulfur, oxygen, nitrogen, and silicone being preferred. Alkyl includes substituted alkyl groups. By "substituted alkyl group" herein is meant an alkyl group further comprising one or more substitution moieties "R", as defined above.
15

By "amino groups" or grammatical equivalents herein is meant $-NH_2$, $-NHR$ and $-NR_2$ groups, with R being as defined herein.

By "nitro group" herein is meant an $-NO_2$ group.
20

By "sulfur containing moieties" herein is meant compounds containing sulfur atoms, including but not limited to, thia-, thio- and sulfo- compounds, thiols (-SH and -SR), and sulfides (-RSR-). By "phosphorus containing moieties" herein is meant compounds containing phosphorus, including, but not limited to, phosphines and phosphates. By "silicon containing moieties" herein is meant compounds
25   containing silicon.

By "ether" herein is meant an -O-R group. Preferred ethers include alkoxy groups, with $-O-(CH_2)_2CH_3$ and $-O-(CH_2)_4CH_3$ being preferred.

30   By "ester" herein is meant a -COOR group.

By "halogen" herein is meant bromine, iodine, chlorine, or fluorine. Preferred substituted alkyls are partially or fully halogenated alkyls such as CF_3, etc.

35   By "aldehyde" herein is meant -RCOH groups.

By "alcohol" herein is meant -OH groups, and alkyl alcohols -ROH.

By "amido" herein is meant -RCONH- or RCONR- groups.

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By "ethylene glycol" herein is meant a $-(O-CH_2-CH_2)_n-$ group, although each carbon atom of the ethylene group may also be singly or doubly substituted, i.e. $-(O-CR_2-CR_2)_n-$, with R as described above. Ethylene glycol derivatives with other heteroatoms in place of oxygen (i.e. $-(N-CH_2-CH_2)_n-$ or $-(S-CH_2-CH_2)_n-$, or with substitution groups) are also preferred.

5

Preferred substitution groups include, but are not limited to, methyl, ethyl, propyl, alkoxy groups such as $-O-(CH_2)_2CH_3$ and $-O-(CH_2)_4CH_3$ and ethylene glycol and derivatives thereof.

Preferred aromatic groups include, but are not limited to, phenyl, naphthyl, naphthalene, anthracene,
10   phenanthroline, pyrole, pyridine, thiophene, porphyrins, and substituted derivatives of each of these, included fused ring derivatives.

In the conductive oligomers depicted herein, when g is 1, B-D is a bond linking two atoms or chemical moieties. In a preferred embodiment, B-D is a conjugated bond, containing overlapping or conjugated
15   π-orbitals.

Preferred B-D bonds are selected from acetylene ($-C\equiv C-$, also called alkyne or ethyne), alkene ($-CH=CH-$, also called ethylene), substituted alkene ($-CR=CR-$, $-CH=CR-$ and $-CR=CH-$), amide ($-NH-CO-$ and $-NR-CO-$ or $-CO-NH-$ and $-CO-NR-$), azo ($-N=N-$), esters and thioesters ($-CO-O-$, $-O-CO-$, $-CS-O-$ and $-O-CS-$) and other conjugated bonds such as ($-CH=N-$, $-CR=N-$, $-N=CH-$ and $-N=CR-$), (
20   $-SiH=SiH-$, $-SiR=SiH-$, $-SiR=SiH-$, and $-SiR=SiR-$), ($-SiH=CH-$, $-SiR=CH-$, $-SiH=CR-$, $-SiR=CR-$, $-CH=SiH-$, $-CR=SiH-$, $-CH=SiR-$, and $-CR=SiR-$). Particularly preferred B-D bonds are acetylene, alkene, amide, and substituted derivatives of these three, and azo. Especially preferred B-D bonds are acetylene, alkene and amide. The oligomer components attached to double bonds may be in the trans
25   or cis conformation, or mixtures. Thus, either B or D may include carbon, nitrogen or silicon. The substitution groups are as defined as above for R.

When g=0 in the Structure 2 conductive oligomer, e is preferably 1 and the D moiety may be carbonyl or a heteroatom moiety as defined above.

30

As above for the Y rings, within any single conductive oligomer, the B-D bonds (or D moieties, when g=0) may be all the same, or at least one may be different. For example, when m is zero, the terminal B-D bond may be an amide bond, and the rest of the B-D bonds may be acetylene bonds. Generally, when amide bonds are present, as few amide bonds as possible are preferable, but in some
35   embodiments all the B-D bonds are amide bonds. Thus, as outlined above for the Y rings, one type of B-D bond may be present in the conductive oligomer within a monolayer as described below, and another type above the monolayer level, to give greater flexibility.

25

In the structures depicted herein, n is an integer from 1 to 50, although longer oligomers may also be used (see for example Schumm et al., Angew. Chem. Int. Ed. Engl. 1994 33(13):1360). Without being bound by theory, it appears that for efficient binding of target analytes on a surface, the binding should occur at a distance from the surface, i.e. the kinetics of binding increase as a function of the

5　distance from the surface, particularly for larger analytes. Accordingly, the length of the conductive oligomer is such that the closest portion of the redox active complex is positioned from about 6Å to about 100Å (although distances of up to 500Å may be used) from the electrode surface, with from about 25Å to about 60Å being preferred. In a preferred embodiment, the length of the conductive oligomer is greater than $(CH_2)_6$ linkers, with greater than $(CH_2)_{10}$ being preferred and greater than about

10　$(CH_2)_{16}$ being particularly preferred. Accordingly, n will depend on the size of the aromatic group, but generally will be from about 1 to about 20, with from about 2 to about 15 being preferred and from about 3 to about 10 being especially preferred.

In the structures depicted herein, m is either 0 or 1. That is, when m is 0, the conductive oligomer may

15　terminate in the B-D bond or D moiety, i.e. the D atom is attached to the component of the redox active complex either directly or via a linker. In some embodiments, there may be additional atoms, such as a linker, attached between the conductive oligomer and the component of the redox active complex to which it is attached. Additionally, as outlined below, the D atom may be a nitrogen atom of a redox active complex, for example an amine of a protein. Alternatively, when m is 1, the conductive oligomer

20　may terminate in Y, an aromatic group, i.e. the aromatic group is attached to the redox active complex or linker.

As will be appreciated by those in the art, a large number of possible conductive oligomers may be utilized. These include conductive oligomers falling within the Structure 1 and Structure 8 formulas, as

25　well as other conductive oligomers, as are generally known in the art, including for example, compounds comprising fused aromatic rings or Teflon®-like oligomers, such as $-(CF_2)_n-$, $-(CHF)_n-$ and $-(CFR)_n-$. See for example, Schumm et al., angew. Chem. Intl. Ed. Engl. 33:1361 (1994);Grosshenny et al., Platinum Metals Rev. 40(1):26-35 (1996); Tour, Chem. Rev. 96:537-553 (1996); Hsung et al., Organometallics 14:4808-4815 (1995; and references cited therein, all of which are expressly

30　incorporated by reference.

Particularly preferred conductive oligomers of this embodiment are depicted below:

Structure 2

35　
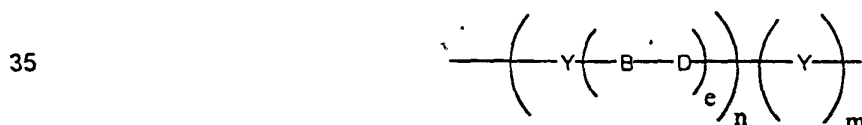
Structure 2 is Structure 1 when g is 1. Preferred embodiments of Structure 2 include: e is zero, Y is pyrole or substituted pyrole; e is zero, Y is thiophene or substituted thiophene; e is zero, Y is furan or

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substituted furan; e is zero, Y is phenyl or substituted phenyl; e is zero, Y is pyridine or substituted pyridine; e is 1, B-D is acetylene and Y is phenyl or substituted phenyl (see Structure 4 below). A preferred embodiment of Structure 2 is also when e is one, depicted as Structure 3 below:
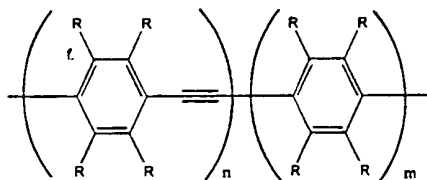
<div align="center">Structure 3</div>

5
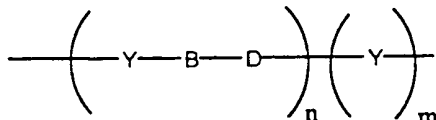
Preferred embodiments of Structure 3 are: Y is phenyl or substituted phenyl and B-D is azo; Y is phenyl
10   or substituted phenyl and B-D is alkene; Y is pyridine or substituted pyridine and B-D is acetylene; Y is thiophene or substituted thiophene and B-D is acetylene; Y is furan or substituted furan and B-D is acetylene; Y is thiophene or furan (or substituted thiophene or furan) and B-D are alternating alkene and acetylene bonds.

15   Most of the structures depicted herein utilize a Structure 3 conductive oligomer. However, any Structure 4 oligomers may be substituted with a Structure 1, 2 or 8 oligomer, or other conducting oligomer, and the use of such Structure 3 depiction is not meant to limit the scope of the invention.

Particularly preferred embodiments of Structure 3 include Structures 4, 5, 6 and 7, depicted below:

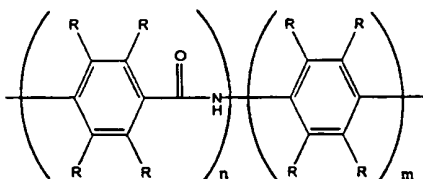20   <div align="center">Structure 4</div>

25

Particularly preferred embodiments of Structure 4 include: n is two, m is one, and R is hydrogen; n is three, m is zero, and R is hydrogen; and the use of R groups to increase solubility.

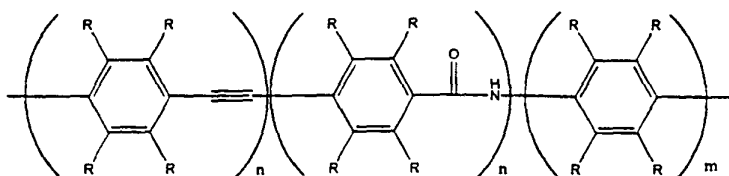<div align="center">Structure 5</div>

30

35   When the B-D bond is an amide bond, as in Structure 5, the conductive oligomers are pseudopeptide oligomers. Although the amide bond in Structure 5 is depicted with the carbonyl to the left, i.e. -CONH-, the reverse may also be used, i.e. -NHCO-. Particularly preferred embodiments of Structure 5 include: n is two, m is one, and R is hydrogen; n is three, m is zero, and R is hydrogen (in this embodiment, the

<div align="center">27</div>

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terminal nitrogen (the D atom) may be the nitrogen of the amino-modified ribose); and the use of R
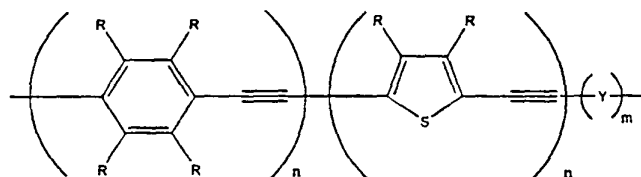groups to increase solubility.

Structure 6

Preferred embodiments of Structure 6 include the first n is two, second n is one, m is zero, and all R
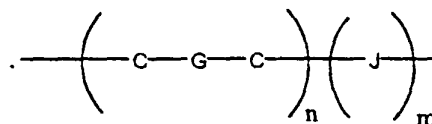groups are hydrogen, or the use of R groups to increase solubility.

Structure 7

Preferred embodiments of Structure 7 include: the first n is three, the second n is from 1-3, with m being
either 0 or 1, and the use of R groups to increase solubility.

In a preferred embodiment, the conductive oligomer has the structure depicted in Structure 8:
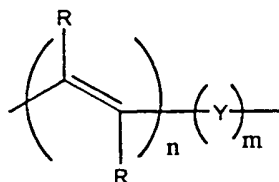
Structure 8

In this embodiment, C are carbon atoms, n is an integer from 1 to 50, m is 0 or 1, J is a heteroatom
selected from the group consisting of nitrogen, silicon, phosphorus, sulfur, carbonyl or sulfoxide, and G
is a bond selected from alkane, alkene or acetylene, such that together with the two carbon atoms the
C-G-C group is an alkene (-CH=CH-), substituted alkene (-CR=CR-) or mixtures thereof (-CH=CR- or -
CR=CH-), acetylene (-C$\equiv$C-), or alkane (-CR$_2$-CR$_2$-, with R being either hydrogen or a substitution
group as described herein). The G bond of each subunit may be the same or different than the G
bonds of other subunits; that is, alternating oligomers of alkene and acetylene bonds could be used,
etc. However, when G is an alkane bond, the number of alkane bonds in the oligomer should be kept
to a minimum, with about six or less sigma bonds per conductive oligomer being preferred. Alkene
bonds are preferred, and are generally depicted herein, although alkane and acetylene bonds may be
substituted in any structure or embodiment described herein as will be appreciated by those in the art.

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In a preferred embodiment, the m of Structure 8 is zero. In a particularly preferred embodiment, m is zero and G is an alkene bond, as is depicted in Structure 9:

Structure 9

5

The alkene oligomer of structure 9, and others depicted herein, are generally depicted in the preferred
10 trans configuration, although oligomers of cis or mixtures of trans and cis may also be used. As above, R groups may be added to alter the packing of the compositions on an electrode, the hydrophilicity or hydrophobicity of the oligomer, and the flexibility, i.e. the rotational, torsional or longitudinal flexibility of the oligomer. n is as defined above.

15 In a preferred embodiment, R is hydrogen, although R may be also alkyl groups and polyethylene glycols or derivatives.

In an alternative embodiment, the conductive oligomer may be a mixture of different types of oligomers, for example of structures 1 and 8.
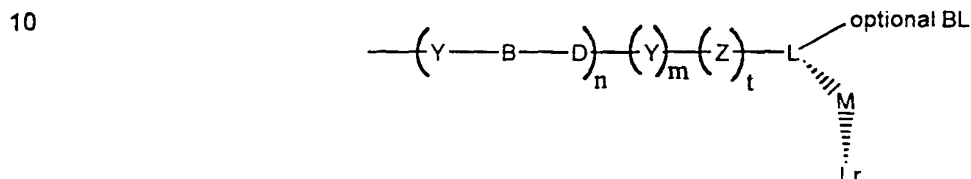20

The conductive oligomers are covalently attached to a component of the redox active complex, either the binding ligand, or the redox active molecule, or both, as is generally outlined in the systems described above. By "covalently attached" herein is meant that two moieties are attached by at least one bond, including sigma bonds, pi bonds and coordination bonds.
25

The method of attachment of the redox active complex to the spacer (also sometimes referred to herein as an attachment linker, which may be either an insulator or conductive oligomer) will generally be done as is known in the art, and will depend on both the composition of the attachment linker and the capture binding ligand. In general, the redox active complexes are attached to the attachment linker through
30 the use of functional groups on each that can then be used for attachment. Preferred functional groups for attachment are amino groups, carboxy groups, oxo groups and thiol groups. These functional groups can then be attached, either directly or indirectly through the use of a linker, sometimes depicted herein as "Z". Linkers are well known in the art; for example, homo-or hetero-bifunctional linkers as are well known (see 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages
35 155-200, incorporated herein by reference). Preferred Z linkers include, but are not limited to, alkyl groups (including substituted alkyl groups and alkyl groups containing heteroatom moieties), with short alkyl groups, esters, amide, amine, epoxy groups and ethylene glycol and derivatives being preferred, with propyl, acetylene, and C_2 alkene being especially preferred. Z may also be a sulfone group, forming sulfonamide linkages.
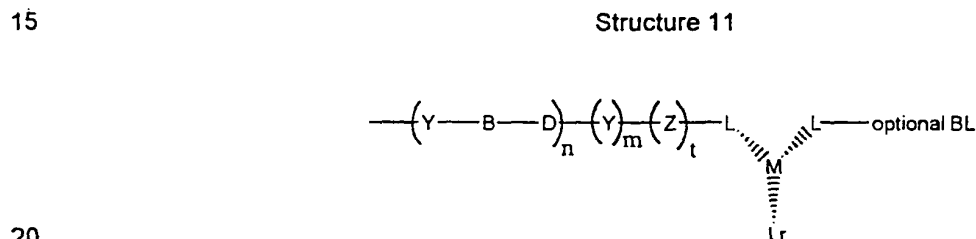
A preferred attachment of redox active molecules that are transition metal complexes utilizes either a transition metal ligand (including coordination atoms) on the terminus of the conductive oligomer, that serves to attach the redox active molecule to the conductive oligomer, as is generally depicted below in Structures 10 and 11. Both Structure 10 and 11 depict a structure 3 conductive oligomer, although

5   other oligomers may be used. Similarly, if a binding ligand is attached (for example as shown in System 4), the metal ligand can either be attached to the binding ligand (i.e. exogeneously added, for example using a Z linker) or can be contributed by the binding ligand itself (for example, using a nitrogen of an amino acid side chain).

Structure 10

10

$$-\left(Y-B-D\right)_n\left(Y\right)_m\left(Z\right)_t-L \overset{\text{optional BL}}{\underset{\substack{\text{M}\\\text{Lr}}}{\Big\langle}}$$

15   Structure 11

$$-\left(Y-B-D\right)_n\left(Y\right)_m\left(Z\right)_t-L \cdots \overset{L——\text{optional BL}}{\underset{\substack{\text{M}\\\text{Lr}}}{}}$$

20
L are the co-ligands, that provide the coordination atoms for the binding of the metal ion. As will be appreciated by those in the art, the number and nature of the co-ligands will depend on the coordination number of the metal ion. Mono-, di- or polydentate co-ligands may be used at any position. Thus, for example, when the metal has a coordination number of six, the L from the terminus of the conductive

25   oligomer, the L contributed from the binding ligand (if present) and r, add up to six. Thus, when the metal has a coordination number of six, r may range from zero (when all coordination atoms are provided by the other ligands) to five, when all the co-ligands are monodentate. Thus generally, r will be from 0 to 8, depending on the coordination number of the metal ion and the choice of the other ligands.

30

In one embodiment, the metal ion has a coordination number of six and both the ligand attached to the conductive oligomer and the ligand either attached or contributed by the binding ligand are at least bidentate; that is, r is preferably zero, one (i.e. the remaining co-ligand is bidentate) or two (two monodentate co-ligands are used).

35

As will be appreciated in the art, the co-ligands can be the same or different.

When one or more of the co-ligands is an organometallic ligand, the ligand is generally attached via one of the carbon atoms of the organometallic ligand, although attachment may be via other atoms for

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heterocyclic ligands. Preferred organometallic ligands include metallocene ligands, including

substituted derivatives and the metalloceneophanes (see page 1174 of Cotton and Wilkenson, supra).

For example, derivatives of metallocene ligands such as methylcyclopentadienyl, with multiple methyl

groups being preferred, such as pentamethylcyclopentadienyl, can be used to increase the stability of

5   the metallocene. In a preferred embodiment, only one of the two metallocene ligands of a metallocene

are derivatized.

In this way, redox active complexes may be attached, including binding ligands comprising proteins,

lectins, nucleic acids, small organic molecules, carbohydrates, etc.

10

A preferred embodiment utilizes proteinaceous binding ligands. As is known in the art, any number of

techniques may be used to attach a proteinaceous binding ligand to an attachment linker, as is outlined

above for the attachment of a redox active complex to the spacer; see also figures 2 and 3. A wide

variety of techniques are known to add moieties to proteins. Similar techniques can be used to add the

15   binding ligand to the redox active molecule, for example as depicted in System 3, 4 or 5, as will be

appreciated by those in the art.

A preferred embodiment utilizes nucleic acids as the binding ligand, with techniques outlined in PCT

US97/20014 being useful for attachment.

20

One end of the attachment linker is linked to the redox active complex, and the other end (although as

will be appreciated by those in the art, it need not be the exact terminus for either) is attached to the

electrode. Thus, any of structures depicted herein may further comprise a redox active complex or

system component effectively as a terminal group.

25

The covalent attachment of the conductive oligomer containing the redox active molecule (and the

attachment of other spacer molecules) may be accomplished in a variety of ways, depending on the

electrode and the conductive oligomer used. Generally, some type of linker is used, as depicted below

as "A" in Structure 12, where X is the conductive oligomer, and the hatched surface is the electrode:

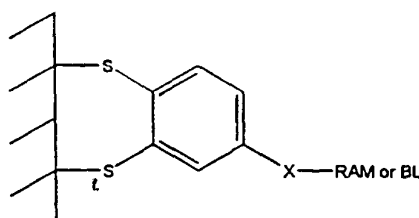30   Structure 12

35
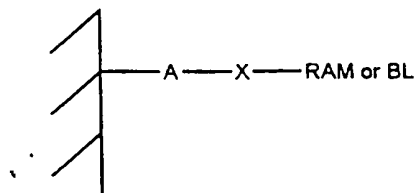
In this embodiment, A is a linker or atom. The choice of "A" will depend in part on the characteristics of

the electrode. Thus, for example, A may be a sulfur moiety when a gold electrode is used.

Alternatively, when metal oxide electrodes are used, A may be a silicon (silane) moiety attached to the

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oxygen of the oxide (see for example Chen et al., Langmuir 10:3332-3337 (1994); Lenhard et al., J.
Electroanal. Chem. 78:195-201 (1977), both of which are expressly incorporated by reference). When
carbon based electrodes are used, A may be an amino moiety (preferably a primary amine; see for
example Deinhammer et al., Langmuir 10:1306-1313 (1994)). Thus, preferred A moieties include, but

5   are not limited to, silane moieties, sulfur moieties (including alkyl sulfur moieties), and amino moieties.
In a preferred embodiment, epoxide type linkages with redox polymers such as are known in the art are
not used.

Although depicted herein as a single moiety, the conductive oligomer (and other spacers) may be

10   attached to the electrode with more than one "A" moiety; the "A" moieties may be the same or different.
Thus, for example, when the electrode is a gold electrode, and "A" is a sulfur atom or moiety, such as
generally depicted below in Structure 13, multiple sulfur atoms may be used to attach the conductive
oligomer to the electrode, such as is generally depicted below in Structures 14, 15 and 16. As will be
appreciated by those in the art, other such structures can be made. In Structures 14, 15 and 16, the A

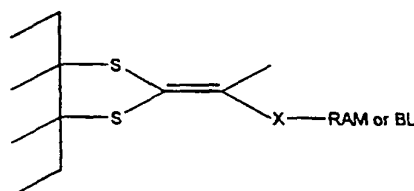15   moiety is just a sulfur atom, but substituted sulfur moieties may also be used.

Structure 14


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Structure 15

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Structure 16

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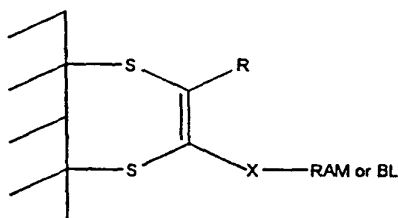
It should also be noted that similar to Structure 16, it may be possible to have a a conductive oligomer
terminating in a single carbon atom with three sulfur moities attached to the electrode.
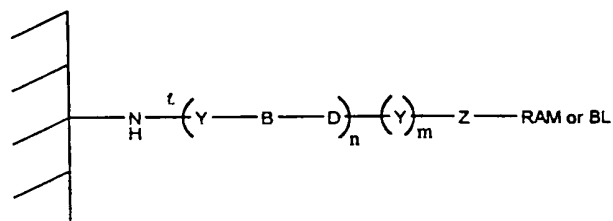
32

In a preferred embodiment, the electrode is a gold electrode, and attachment is via a sulfur linkage as is well known in the art, i.e. the A moiety is a sulfur atom or moiety. Although the exact characteristics of the gold-sulfur attachment are not known, this linkage is considered covalent for the purposes of this invention. A representative structure is depicted in Structure 17. Structure 17 depicts the "A" linker as

5   comprising just a sulfur atom, although additional atoms may be present (i.e. linkers from the sulfur to the conductive oligomer or substitution groups).

Structure 17

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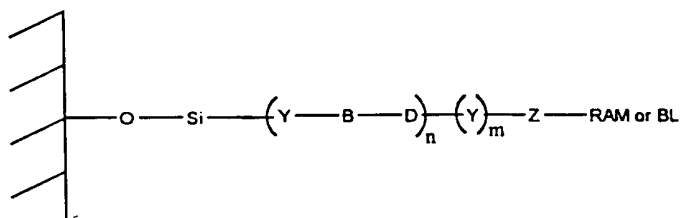$$S-\left(Y-B-D\right)_n\left(Y\right)_m-Z---RAM\ or\ BL$$
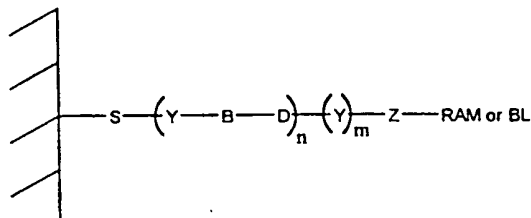
15   In a preferred embodiment, the electrode is a carbon electrode, i.e. a glassy carbon electrode, and attachment is via a nitrogen of an amine group. A representative structure is depicted in Structure 18. Again, additional atoms may be present, i.e. Z type linkers.

Structure 18

20

$$\underset{H}{N}-\left(Y-B-D\right)_n\left(Y\right)_m-Z---RAM\ or\ BL$$

25

Structure 19

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$$O-Si-\left(Y-B-D\right)_n\left(Y\right)_m-Z---RAM\ or\ BL$$

In Structure 19, the oxygen atom is from the oxide of the metal oxide electrode. The Si atom may also

35   contain other atoms, i.e. be a silicon moiety containing substitution groups.

Thus, in a preferred embodiment, electrodes are made that comprise conductive oligomers attached to redox active complexes for the purposes of hybridization assays, as is more fully described herein. As will be appreciated by those in the art, electrodes can be made that have a single species of binding

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ligand, i.e. for the detection of a single target analyte, or multiple binding ligand species, i.e. for the detection of multiple target analytes.

In addition, as outlined herein, the use of a solid support such as an electrode enables the use of these
5   assays in an array form. The use of arrays such as oligonucleotide arrays are well known in the art, and similar systems can be built herein. In addition, techniques are known for "addressing" locations within an electrode array and for the surface modification of electrodes. Thus, in a preferred embodiment, arrays of different binding ligands are laid down on the array of electrodes, each of which are covalently attached to the electrode via a conductive linker. In this embodiment, the number of
10   different species of analytes may vary widely, from one to thousands, with from about 4 to about 100,000 being preferred, and from about 10 to about 10,000 being particularly preferred.

In a preferred embodiment, the electrode further comprises a passavation agent, preferably in the form of a monolayer on the electrode surface. The efficiency of binding may increase when the target
15   analyte is at a distance from the electrode, and non-specific binding is decreased when a monolayer is used. A passavation agent layer facilitates the maintenance of the target analyte away from the electrode surface. In addition, a passavation agent serves to keep charge carriers away from the surface of the electrode. Thus, this layer helps to prevent electrical contact between the electrodes and the electron transfer moieties, or between the electrode and charged species within the solvent. Such
20   contact can result in a direct "short circuit" or an indirect short circuit via charged species which may be present in the sample. Accordingly, the monolayer of passavation agents is preferably tightly packed in a uniform layer on the electrode surface, such that a minimum of "holes" exist. Alternatively, the passavation agent may not be in the form of a monolayer, but may be present to help the packing of the conductive oligomers or other characteristics.
25

The passavation agents thus serve as a physical barrier to block solvent accesibility to the electrode. As such, the passavation agents themselves may in fact be either (1) conducting or (2) nonconducting, i.e. insulating, molecules. Thus, in one embodiment, the passavation agents are conductive oligomers, as described herein, with or without a terminal group to block or decrease the transfer of charge to the
30   electrode. Other passavation agents include oligomers of $-(CF_2)_n-$, $-(CHF)_n-$ and $-(CFR)_n-$. In a preferred embodiment, the passavation agents are insulator moieties.

An "insulator" is a substantially nonconducting oligomer, preferably linear. By "substantially nonconducting" herein is meant that the rate of electron transfer through the insulator is the rate limiting
35   step of the transfer reaction. Stated differently, the electrical resistance of the insulator is higher than the electrical resistance of the rest of the system. The rate of electron transfer through the insulator is preferrably slower than the rate through the conductive oligomers described herein. It should be noted however that even oligomers generally considered to be insulators still may transfer electrons, albeit at a slow rate.

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In a preferred embodiment, the insulators have a conductivity, S, of about $10^{-7} \ \Omega^{-1}cm^{-1}$ or lower, with less than about $10^{-8} \ \Omega^{-1}cm^{-1}$ being preferred. See generally Gardner et al., supra.

5   Generally, insulators are alkyl or heteroalkyl oligomers or moieties with sigma bonds, although any particular insulator molecule may contain aromatic groups or one or more conjugated bonds. By "heteroalkyl" herein is meant an alkyl group that has at least one heteroatom, i.e. nitrogen, oxygen, sulfur, phosphorus, silicon or boron included in the chain. Alternatively, the insulator may be quite similar to a conductive oligomer with the addition of one or more heteroatoms or bonds that serve to inhibit or slow, preferably substantially, electron transfer.

10

The passavation agents, including insulators, may be substituted with R groups as defined herein to alter the packing of the moieties or conductive oligomers on an electrode, the hydrophilicity or hydrophobicity of the insulator, and the flexibility, i.e. the rotational, torsional or longitudinal flexibility of the insulator. For example, branched alkyl groups may be used. In addition, the terminus of the

15 passavation agent, including insulators, may contain an additional group to influence the exposed surface of the monolayer. For example, there may be negatively charged groups on the terminus to form a negatively charged surface to repel negatively charged species from non-specifically binding. Similarly, for example as depicted in System 1, hydrophobic groups can be used to attract hydrophobic analytes, etc. Preferred passavation agent terminal groups include $-NH_2$, $-OH$, $-COOH$, $-CH_3$,

20 trimethylsilyl (TMS) and (poly)ethylene glycol, with the latter being particularly preferred.

The length of the passavation agent will vary as needed. As outlined above, it appears that binding is more efficient at a distance from the surface. Thus, the length of the passavation agents is similar to the length of the conductive oligomers, as outlined above. In addition, the conductive oligomers may be

25 basically the same length as the passavation agents or longer than them, resulting in the binding ligands being more accessible to the solvent for binding of target analytes.

The monolayer may comprise a single type of passavation agent, including insulators, or different types.

30

Suitable insulators are known in the art, and include, but are not limited to, $-(CH_2)_n-$, $-(CRH)_n-$, and $-(CR_2)_n-$, ethylene glycol or derivatives using other heteroatoms in place of oxygen, i.e. nitrogen or sulfur (sulfur derivatives are not preferred when the electrode is gold).

35 The passavation agents are generally attached to the electrode in the same manner as the conductive oligomer, and may use the same "A" linker as defined above.

The compositions of the invention are generally synthesized as outlined below, generally utilizing techniques well known in the art.

35

The compositions may be made in several ways. A preferred method first synthesizes a conductive oligomer attached to the binding ligand or redox active molecule, followed by attachment to the electrode. The second component of the redox active complex may be added prior to attachment to the electrode or after. Alternatively, the redox active complex may be made and then the completed

5   conductive oligomer added, followed by attachment to the electrode. Alternatively, the conductive oligomer and monolayer (if present) are attached to the electrode first, followed by attachment of the other components.

In a preferred embodiment, conductive oligomers are covalently attached via sulfur linkages to the

10   electrode. However, surprisingly, traditional protecting groups for use of attaching molecules to gold electrodes are generally not ideal for use in both synthesis of the compositions described herein and inclusion in biomolecule synthetic reactions. Accordingly, alternate methods for the attachment of conductive oligomers to gold electrodes, utilizing unusual protecting groups, including ethylpyridine, and trimethylsilylethyl as is described in PCT US97/20014. Briefly, in a preferred embodiment, the subunit

15   of the conductive oligomer which contains the sulfur atom for attachment to the electrode is protected with an ethyl-pyridine or trimethylsilylethyl group. For the former, this is generally done by contacting the subunit containing the sulfur atom (preferably in the form of a sulfhydryl) with a vinyl pyridine group or vinyl trimethylsilylethyl group under conditions whereby an ethylpyridine group or trimethylsilylethyl group is added to the sulfur atom.

20

This subunit also generally contains a functional moiety for attachment of additional subunits, and thus additional subunits are attached to form the conductive oligomer. The conductive oligomer is then attached to a component of the redox active complex. The protecting group is then removed and the sulfur-gold covalent attachment is made. Alternatively, all or part of the conductive oligomer is made,

25   and then either a subunit containing a protected sulfur atom is added, or a sulfur atom is added and then protected. The conductive oligomer is then attached to a component of the redox active complex. Alternatively, the conductive oligomer attached to the redox active complex component, and then either a subunit containing a protected sulfur atom is added, or a sulfur atom is added and then protected. Alternatively, the ethyl pyridine protecting group may be used as above, but removed after one or more

30   steps and replaced with a standard protecting group like a disulfide. Thus, the ethyl pyridine or trimethylsilylethyl group may serve as the protecting group for some of the synthetic reactions, and then removed and replaced with a traditional protecting group.

By "subunit" of a conductive polymer herein is meant at least the moiety of the conductive oligomer to

35   which the sulfur atom is attached, although additional atoms may be present, including either functional groups which allow the addition of additional components of the conductive oligomer, or additional components of the conductive oligomer. Thus, for example, when Structure 1 oligomers are used, a subunit comprises at least the first Y group.

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A preferred method comprises 1) adding an ethyl pyridine or trimethylsilylethyl protecting group to a sulfur atom attached to a first subunit of a conductive oligomer, generally done by adding a vinyl pyridine or trimethylsilylethyl group to a sulfhydryl; 2) adding additional subunits to form the conductive oligomer; 3) adding at least a first component of the redox active complex to the conductive oligomer; 4)
5   adding additional components as necessary; and 5) attaching the conductive oligomer to the gold electrode.

The above method may also be used to attach passavation molecules to a gold electrode.

10   In a preferred embodiment, a monolayer of passavation agents is added to the electrode. Generally, the chemistry of addition is similar to or the same as the addition of conductive oligomers to the electrode, i.e. using a sulfur atom for attachment to a gold electrode, etc. Compositions comprising monolayers in addition to the conductive oligomers covalently attached to components of the redox active complex may be made in at least one of five ways: (1) addition of the monolayer, followed by
15   subsequent addition of the conductive oligomer- redox activecomplex; (2) addition of the conductive oligomer-redox active complex followed by addition of the monolayer; (3) simultaneous addition of the monolayer and conductive oligomer-redox active complex; (4) formation of a monolayer (using any of 1, 2 or 3) which includes conductive oligomers which terminate in a functional moiety suitable for attachment of a redox active complex; or (5) formation of a monolayer which includes conductive
20   oligomers which terminate in a functional moiety suitable for synthesis, i.e. the redox active complex (for example, the binding ligand) is synthesized on the surface of the monolayer as is known in the art. Such suitable functional moieties include, but are not limited to, nucleosides, amino groups, carboxyl groups, protected sulfur moieties, or hydroxyl groups for phosphoramidite additions.

25   As will be appreciated by those in the art, electrodes may be made that have any combination of components. Thus, a variety of different conductive oligomers or passavation agents may be used on a single electrode.

Once made, the compositions find use in a number of applications, as described herein.
30

The compositions of the invention thus comprise assay complexes comprising a target analyte bound to a redox active complex, wherein the complex comprises a binding ligand and a redox active molecule. In a preferred embodiment, the ligand-analyte interaction is such that the environment of the redox active molecule changes sufficiently upon binding to alter a measurable redox property of the redox
35   active molecule. For example, in antibody-antigen complexes, enzyme-substrate (or inhibitor) complexes, other protein-protein interactions, etc., the redox active molecule is generally located within or adjacent to the binding site or active site of the interaction, such that upon binding, the environment of the redox active molecule changes. This may be due to a conformational change, a "shielding" of the redox active molecule, new solvent accessibility of the redox active molecule, etc. Preferably, the redox

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active molecule is placed such that it does not inhibit the ligand-target binding but is affected by it. In general, the RAM is generally within less than 50Å of the target analyte, with less than about 25Å being preferred, and less than 6-10 Å being particularly preferred.

5   In general, changes in faradaic impedance are due to changes in the rate of electron transfer between the RAM, generally through the conductive oligomer, to the electrode. As predicted by semiclassical theory, changes in the rate of electron transfer can be due to changes in the intervening medium (conceptually, changes in H_{AB}), changes in nuclear reorganization energy, λ (the major component of which is the solvent reorganization energy), changes in the driving force (-$\Delta G°$; which is generally a

10   function of changes in the input signal, rather than changes in the system as a result of analyte binding), changes in distance, according to the following equation:

$$k_{ET} = (4\pi^3/h^2\lambda k_B T)^{1/2}(H_{AB})^2 \exp[(-\Delta G° + \lambda)^2/\lambda k_B T]$$

15   Thus, as generally discussed herein, changes in faradaic impedance are generally determined by evaluating the changes in the rate and/or quantity of electron transfer between the RAM and the electrode. Accordingly, changes in faradaic impedance are done by initiating electron transfer, generally both in the absence and presence of the target analyte, and evaluating the generated signal, which will be characteristic of either the absence or presence of the target analyte. In some

20   embodiments, for example in system 8, there may be little or no electron transfer in the absence of the analyte. Other systems rely on changes in electron transfer rate or quantity on the basis of the presence or absence of the target analyte.

Electron transfer is generally initiated electronically, with the application of at least a first input signal,

25   with voltage being preferred. A potential is applied to the assay complex. Precise control and variations in the applied potential can be via a potentiostat and either a three electrode system (one reference, one sample (or working) and one counter electrode) or a two electrode system (one sample and one counter electrode). This allows matching of applied potential to peak potential of the system which depends in part on the choice of RAMs and in part on the conductive oligomer used, the

30   composition and integrity of the monolayer, and what type of reference electrode is used. As described herein, ferrocene is a preferred RAM.

In a preferred embodiment, a co-reductant or co-oxidant (collectively, co-redoxant) is used, as an additional electron source or sink. See generally Sato et al., Bull. Chem. Soc. Jpn 66:1032 (1993);

35   Uosaki et al., Electrochimica Acta 36:1799 (1991); and Alleman et al., J. Phys. Chem 100:17050 (1996); all of which are incorporated by reference. This finds use when DC detection modes are used, or slow AC, i.e. non-diffusion limited AC.

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In a preferred embodiment, an input electron source in solution is used in the initiation of electron transfer, preferably when initiation and detection are being done using DC current or at AC frequencies where diffusion is not limiting. In general, as will be appreciated by those in the art, preferred embodiments utilize monolayers that contain a minimum of "holes", such that short-circuiting of the

5   system is avoided. This may be done in several general ways. In a preferred embodiment, an input electron source is used that has a lower or similar redox potential than the RAM of the assay complex. Thus, at voltages above the redox potential of the input electron source, both the RAM and the input electron source are oxidized and can thus donate electrons; the RAM donates an electron to the electrode and the input source donates to the RAM. For example, ferrocene, as a RAM attached to the

10   compositions of the invention as described in the examples, has a redox potential of roughly 200 mV in aqueous solution (which can change significantly depending on what the ferrocene is bound to, the manner of the linkage and the presence of any substitution groups). Ferrocyanide, an electron source, has a redox potential of roughly 200 mV as well (in aqueous solution). Accordingly, at or above voltages of roughly 200 mV, ferrocene is converted to ferricenium, which then transfers an electron to

15   the electrode. Now the ferricyanide can be oxidized to transfer an electron to the RAM. In this way, the electron source (or co-reductant) serves to amplify the signal generated in the system, as the electron source molecules rapidly and repeatedly donate electrons to the RAM attached to the assay complex. The rate of electron donation or acceptance will be limited by the rate of diffusion of the co-reductant, the electron transfer between the co-reductant and the RAM, which in turn is affected by the

20   concentration and size, etc.

Alternatively, input electron sources that have lower redox potentials than the RAM are used. At voltages less than the redox potential of the RAM, but higher than the redox potential of the electron source, the input source such as ferrocyanide is unable to be oxided and thus is unable to donate an

25   electron to the RAM; i.e. no electron transfer occurs. Once ferrocene is oxidized, then there is a pathway for electron transfer.

In an alternate preferred embodiment, an input electron source is used that has a higher redox potential than the RAM of the label probe. For example, luminol, an electron source, has a redox potential of

30   roughly 720 mV. At voltages higher than the redox potential of the RAM, but lower than the redox potential of the electron source, i.e. 200 - 720 mV, the ferrocene is oxided, and transfers a single electron to the electrode via the conductive oligomer. However, the RAM is unable to accept any electrons from the luminol electron source, since the voltages are less than the redox potential of the luminol. However, at or above the redox potential of luminol, the luminol then transfers an electron to

35   the RAM, allowing rapid and repeated electron transfer. In this way, the electron source (or co-reductant) serves to amplify the signal generated in the system, as the electron source molecules rapidly and repeatedly donate electrons to the RAM of the assay complex.

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Luminol has the added benefit of becoming a chemiluminiscent species upon oxidation (see Jirka et al., Analytica Chimica Acta 284:345 (1993)), thus allowing photo-detection of electron transfer from the RAM to the electrode. Thus, as long as the luminol is unable to contact the electrode directly, i.e. in the presence of the SAM such that there is no efficient electron transfer pathway to the electrode, luminol

5 can only be oxidized by transferring an electron to the RAM on the assay complex. When the RAM is not present, luminol is not significantly oxidized, resulting in a low photon emission and thus a low (if any) signal from the luminol. In the presence of the target, a much larger signal is generated. Thus, the measure of luminol oxidation by photon emission is an indirect measurement of the ability of the RAM to donate electrons to the electrode. Furthermore, since photon detection is generally more

10 sensitive than electronic detection, the sensitivity of the system may be increased. Initial results suggest that luminescence may depend on hydrogen peroxide concentration, pH, and luminol concentration, the latter of which appears to be non-linear.

Suitable electron source molecules are well known in the art, and include, but are not limited to,

15 ferricyanide, and luminol.

Alternatively, output electron acceptors or sinks could be used, i.e. the above reactions could be run in reverse, with the RAM such as a metallocene receiving an electron from the electrode, converting it to the metallicenium, with the output electron acceptor then accepting the electron rapidly and repeatedly.

20 In this embodiment, cobalticenium is the preferred RAM.

Changes in the faradaic impedance of the system, e.g. differences in the rate or quantity of electron transfer, can be detected in a variety of ways. A variety of detection methods may be used, including, but not limited to, optical detection (as a result of spectral changes upon changes in redox states),

25 which includes fluorescence, phosphorescence, luminiscence, chemiluminescence, electrochemiluminescence, and refractive index; and electronic detection, including, but not limited to, amperommetry, voltammetry, capacitance and impedance. These methods include time or frequency dependent methods based on AC or DC currents, pulsed methods, lock-in techniques, filtering (high pass, low pass, band pass), and time-resolved techniques including time-resolved fluoroscence.

30

In one embodiment, the efficient transfer of electrons from the RAM to the electrode results in stereotyped changes in the redox state of the RAM. With many RAMs, including the complexes of ruthenium containing bipyridine, pyridine and imidazole rings, these changes in redox state are associated with changes in spectral properties. Significant differences in absorbance are observed

35 between reduced and oxidized states for these molecules. See for example Fabbrizzi et al., Chem. Soc. Rev. 1995 pp197-202). These differences can be monitored using a spectrophotometer or simple photomultiplier tube device.

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In this embodiment, possible electron donors and acceptors include all the derivatives listed above for photoactivation or initiation. Preferred electron donors and acceptors have characteristically large spectral changes upon oxidation and reduction resulting in highly sensitive monitoring of electron transfer. Such examples include $Ru(NH_3)_4py$ and $Ru(bpy)_2im$ as preferred examples. It should be
5   understood that only the donor or acceptor that is being monitored by absorbance need have ideal spectral characteristics.

In a preferred embodiment, the electron transfer is detected fluorometrically. Numerous transition metal complexes, including those of ruthenium, have distinct fluorescence properties. Therefore, the
10   change in redox state of the electron donors and electron acceptors attached to the redox active complex can be monitored very sensitively using fluorescence, for example with $Ru(4,7$-biphenyl$_2$-phenanthroline)$_3^{2+}$. The production of this compound can be easily measured using standard fluorescence assay techniques. For example, laser induced fluorescence can be recorded in a standard single cell fluorimeter, a flow through "on-line" fluorimeter (such as those attached to a
15   chromatography system) or a multi-sample "plate-reader" similar to those marketed for 96-well immuno assays.

Alternatively, fluorescence can be measured using fiber optic sensors with binding ligands in solution or attached to the fiber optic. Fluorescence is monitored using a photomultiplier tube or other light
20   detection instrument attached to the fiber optic. The advantage of this system is the extremely small volumes of sample that can be assayed.

In addition, scanning fluorescence detectors such as the FluorImager sold by Molecular Dynamics are ideally suited to monitoring the fluorescence of modified molecules arrayed on solid surfaces. The
25   advantage of this system is the large number of electron transfer probes that can be scanned at once using chips covered with thousands of distinct binding ligands.

Many transition metal complexes display fluorescence with large Stokes shifts. Suitable examples include bis- and trisphenanthroline complexes and bis- and trisbipyridyl complexes of transition metals
30   such as ruthenium (see Juris, A., Balzani, V., et. al. Coord. Chem. Rev., V. 84, p. 85-277, 1988). Preferred examples display efficient fluorescence (reasonably high quantum yields) as well as low reorganization energies. These include $Ru(4,7'$-biphenyl$_2$-phenanthroline)$_3^{2+}$, $Ru(4,4'$-diphenyl-2,2'-bipyridine)$_3^{2+}$ and platinum complexes (see Cummings et al., J. Am. Chem. Soc. 118:1949-1960 (1996), incorporated by reference). Alternatively, a *reduction* in fluorescence associated with hybridization can
35   be measured using these systems.

In a further embodiment, electrochemiluminescence is used as the basis of the electron transfer detection. With some RAMs such as $Ru^{2+}(bpy)_3$, direct luminescence accompanies excited state decay. Changes in this property are associated with ligand binding and can be monitored with a simple

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photomultiplier tube arrangement (see Blackburn, G. F. *Clin. Chem.* 37: 1534-1539 (1991); and Juris et al., supra.

5   In a preferred embodiment, electronic detection is used, including amperommetry, voltammetry, capacitance, and impedence. Suitable techniques include, but are not limited to, electrogravimetry; coulometry (including controlled potential coulometry and constant current coulometry); voltametry (cyclic voltametry, pulse voltametry (normal pulse voltametry, square wave voltametry, differential pulse voltametry, Osteryoung square wave voltametry, and coulostatic pulse techniques); stripping analysis (aniodic stripping analysis, cathiodic stripping analysis, square wave stripping voltammetry);

10   conductance measurements (electrolytic conductance, direct analysis); time-dependent electrochemical analyses (chronoamperometry, chronopotentiometry, cyclic chronopotentiometry and amperometry, AC polography, chronogalvametry, and chronocoulometry); AC impedance measurement; capacitance measurement; AC voltametry; and photoelectrochemistry.

15   In a preferred embodiment, monitoring electron transfer is via amperometric detection. This method of detection involves applying a potential (as compared to a separate reference electrode) between the complex-conjugated electrode and a reference (counter) electrode in the sample containing target genes of interest. Electron transfer of differing efficiencies is induced in samples in the presence or absence of target analytes; that is, the presence or absence of the target analyte can result in different

20   currents.

The device for measuring electron transfer amperometrically involves sensitive current detection and includes a means of controlling the voltage potential, usually a potentiostat. This voltage is optimized with reference to the potential of the electron donating complex on the label probe. Possible electron

25   donating complexes include those previously mentioned with complexes of iron, osmium, platinum, cobalt, rhenium and ruthenium being preferred and complexes of iron being most preferred.

In a preferred embodiment, alternative electron detection modes are utilized. For example, potentiometric (or voltammetric) measurements involve non-faradaic (no net current flow) processes

30   and are utilized traditionally in pH and other ion detectors. Similar sensors are used to monitor electron transfer between the RAM and the electrode. In addition, other properties of insulators (such as resistance) and of conductors (such as conductivity, impedance and capicitance) could be used to monitor electron transfer between RAM and the electrode. Finally, any system that generates a current (such as electron transfer) also generates a small magnetic field, which may be monitored in some

35   embodiments.

It should be understood that one benefit of the fast rates of electron transfer observed in the compositions of the invention is that time resolution can greatly enhance the signal-to-noise results of monitors based on absorbance, fluorescence and electronic current. The fast rates of electron transfer

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of the present invention result both in high signals and stereotyped delays between electron transfer

initiation and completion. By amplifying signals of particular delays, such as through the use of pulsed

initiation of electron transfer and "lock-in" amplifiers of detection, and Fourier transforms.

5   In a preferred embodiment, electron transfer is initiated using alternating current (AC) methods.
Without being bound by theory, it appears that RAMs, bound to an electrode, generally respond
similarly to an AC voltage across a circuit containing resistors and capacitors. Basically, any methods
which enable the determination of the nature of these complexes, which act as a resistor and capacitor,
can be used as the basis of detection. Surprisingly, traditional electrochemical theory, such as

10   exemplified in Laviron et al., J. Electroanal. Chem. 97:135 (1979) and Laviron et al., J. Electroanal.
Chem. 105:35 (1979), both of which are incorporated by reference, do not accurately model the
systems described herein, except for very small E_{AC} (less than 10 mV) and relatively large numbers of
molecules. That is, the AC current (I) is not accurately described by Laviron's equation. This may be
due in part to the fact that this theory assumes an unlimited source and sink of electrons, which is not

15   true in the present systems.

Accordingly, alternate equations were developed, using the Nernst equation and first principles to
develop a model which more closely simulates the results. This was derived as follows. The Nernst
equation, Equation 1 below, describes the ratio of oxidized (O) to reduced (R) molecules (number of

20   molecules = n) at any given voltage and temperature, since not every molecule gets oxidized at the
same oxidation potential.

Equation 1

$$E_{DC} = E_0 + \frac{RT}{nF} \ln\frac{[O]}{[R]} \tag{1}$$

E_{DC} is the electrode potential, E_0 is the formal potential of the metal complex, R is the gas constant, T is
the temperature in degrees Kelvin, n is the number of electrons transferred, F is faraday's constant, [O]

25   is the concentration of oxidized molecules and [R] is the concentration of reduced molecules.

The Nernst equation can be rearranged as shown in Equations 2 and 3:

Equation 2

$$E_{DC} - E_0 = \frac{RT}{nF} \ln\frac{[O]}{[R]} \tag{2}$$

E_{DC} is the DC component of the potential.

43

Equation 3

$$\exp^{\frac{nF}{RT}(E_{DC} - E_0)} = \frac{[O]}{[R]} \qquad (3)$$

5

Equation 3 can be rearranged as follows, using normalization of the concentration to equal 1 for simplicity, as shown in Equations 4, 5 and 6. This requires the subsequent multiplication by the total number of molecules.

10

Equation 4 $[O] + [R] = 1$

Equation 5 $[O] = 1 - [R]$

Equation 6 $[R] = 1 - [O]$

Plugging Equation 5 and 6 into Equation 3, and the fact that nF/RT equals 38.9 V^{-1}, for n=1, gives

15   Equations 7 and 8, which define [O] and [R], respectively:

Equation 7

$$[O] = \frac{\exp^{38.9(E-E_0)}}{1 + \exp^{38.9(E-E_0)}} \qquad (4)$$

20

Equation 8

$$[R] = \frac{1}{1 + \exp^{38.9(E - E_0)}} \qquad (5)$$

25

Taking into consideration the generation of an AC faradaic current, the ratio of [O]/[R] at any given potential must be evaluated. At a particular E_{DC} with an applied E_{AC}, as is generally described herein, at the apex of the E_{AC} more molecules will be in the oxidized state, since the voltage on the surface is now

30     $(E_{DC} + E_{AC})$; at the bottom, more will be reduced since the voltage is lower. Therefore, the AC current at a given E_{DC} will be dictated by both the AC and DC voltages, as well as the shape of the Nernstian curve. Specifically, if the number of oxidized molecules at the bottom of the AC cycle is subtracted from the amount at the top of the AC cycle, the total change in a given AC cycle is obtained, as is generally described by Equation 9. Dividing by 2 then gives the AC amplitude.

35   Equation 9

$$i_{AC} = \frac{(\text{electrons at } [E_{DC} + E_{AC}]) - (\text{electrons at } [E_{DC} - E_{AC}])}{2}$$

40   Equation 10 thus describes the AC current which should result:

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Equation 10

$$i_{AC} = C_0 \, F\omega \, \tfrac{1}{2} \, ([O]_{E_{DC} + E_{AC}} - [O]_{E_{DC} - E_{AC}}) \quad (6)$$

5   As depicted in Equation 11, the total AC current will be the number of redox molecules C), times faraday's constant (F), times the AC frequency (ω), times 0.5 (to take into account the AC amplitude), times the ratios derived above in Equation 7. The AC voltage is approximated by the average amplitude, $E_{AC}2/\pi$.

10   Equation 11

$$C = \frac{C_0 \, F\omega}{2} \left(\frac{\exp^{38.9 \, [E_{DC} + \frac{2E_{AC}}{\pi} - E_0]}}{1 + \exp^{38.9 \, [E_{DC} + \frac{2E_{AC}}{\pi} - E_0]}} \right) - \frac{\exp^{38.9 \, [E_{DC} - \frac{2E_{AC}}{\pi} - E_0]}}{1 + \exp^{38.9 \, [E_{DC} - \frac{2E_{AC}}{\pi} - E}} \quad (7)$$

15

However, Equation 11 does not incorporate the effect of electron transfer rate nor of instrument factors including input impedance and stray capacitance. Electron transfer rate is important when the rate is close to or lower than the applied frequency. Thus, the true i_{AC} should be a function of all three, as

20   depicted in Equation 12.

Equation 12

$$i_{AC} = f(\text{Nernst factors}) f(k_{ET}) f(\text{instrument factors})$$

25   These equations can be used to model and predict the expected AC currents in systems which use input signals comprising both AC and DC components. As outlined above, traditional theory surprisingly does not model these systems at all, except for very low voltages.

In general, non-specifically bound analytes can show differences in impedance (i.e. higher impedances) than when the analytes are specifically bound. In a preferred embodiment, the non-specifically bound
30   material is washed away, resulting in an effective impedance of infinity. Thus, AC detection gives several advantages as is generally discussed below, including an increase in sensitivity, and the ability to "filter out" background noise. In particular, changes in impedance (including, for example, bulk impedance) as between non-specific binding of target analytes and target-specific assay complex
35   formation may be monitored.

Accordingly, when using AC initiation and detection methods, the frequency response of the system changes as a result of the presence of the RAM. By "frequency response" herein is meant a modification of signals as a result of electron transfer between the electrode and the RAM. This

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modification is different depending on signal frequency. A frequency response includes AC currents at one or more frequencies, phase shifts, DC offset voltages, faradaic impedance, etc.

Once the assay complex including the target analyte is made, a first input electrical signal is then
5   applied to the system, preferably via at least the sample electrode (containing the complexes of the invention) and the counter electrode, to initiate electron transfer between the electrode and the RAM. Three electrode systems may also be used, with the voltage applied to the reference and working electrodes. The first input signal comprises at least an AC component. The AC component may be of variable amplitude and frequency. Generally, for use in the present methods, the AC amplitude ranges
10   from about 1 mV to about 1.1 V, with from about 10 mV to about 800 mV being preferred, and from about 10 mV to about 300 mV being especially preferred. The AC frequency ranges from about 10 Hz to about 100 KHz, with from about 10 Hz to about 10 MHz being preferred, and from about 100 Hz to about 20 MHz being especially preferred.

15   The use of combinations of AC and DC signals gives a variety of advantages, including surprising sensitivity and signal maximization.

In a preferred embodiment, the first input signal comprises a DC component and an AC component. That is, a DC offset voltage between the sample and counter electrodes is swept through the
20   electrochemical potential of the RAM (for example, when ferrocene is used, the sweep is generally from 0 to 500 mV) (or alternatively, the working electrode is grounded and the reference electrode is swept from 0 to -500 mV). The sweep is used to identify the DC voltage at which the maximum response of the system is seen. This is generally at or about the electrochemical potential of the RAM. Once this voltage is determined, either a sweep or one or more uniform DC offset voltages may be used. DC
25   offset voltages of from about -1 V to about +1.1 V are preferred, with from about -500 mV to about +800 mV being especially preferred, and from about -300 mV to about 500 mV being particularly preferred. In a preferred embodiment, the DC offset voltage is not zero. On top of the DC offset voltage, an AC signal component of variable amplitude and frequency is applied. If the RAM is present, and can respond to the AC perturbation, an AC current will be produced due to electron transfer between the
30   electrode and the RAM.

For defined systems, it may be sufficient to apply a single input signal to differentiate between the presence and absence of the target analyte. Alternatively, a plurality of input signals are applied. As outlined herein, this may take a variety of forms, including using multiple frequencies, multiple DC offset
35   voltages, or multiple AC amplitudes, or combinations of any or all of these.

Thus, in a preferred embodiment, multiple DC offset voltages are used, although as outlined above, DC voltage sweeps are preferred. This may be done at a single frequency, or at two or more frequencies .

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In a preferred embodiment, the AC amplitude is varied. Without being bound by theory, it appears that increasing the amplitude increases the driving force. Thus, higher amplitudes, which result in higher overpotentials give faster rates of electron transfer. Thus, generally, the same system gives an improved response (i.e. higher output signals) at any single frequency through the use of higher

5   overpotentials at that frequency. Thus, the amplitude may be increased at high frequencies to increase the rate of electron transfer through the system, resulting in greater sensitivity. In addition, this may be used, for example, to induce responses in slower systems such as those that do not possess optimal spacing configurations.

10   In a preferred embodiment, measurements of the system are taken at at least two separate amplitudes or overpotentials, with measurements at a plurality of amplitudes being preferred. As noted above, changes in response as a result of changes in amplitude may form the basis of identification, calibration and quantification of the system. In addition, one or more AC frequencies can be used as well.

15   In a preferred embodiment, the AC frequency is varied. At different frequencies, different molecules respond in different ways. As will be appreciated by those in the art, increasing the frequency generally increases the output current. However, when the frequency is greater than the rate at which electrons may travel between the electrode and the RAM higher frequencies result in a loss or decrease of output signal. At some point, the frequency will be greater than the rate of electron transfer between the RAM

20   and the electrode, and then the output signal will also drop.

In one embodiment, detection utilizes à single measurement of output signal at a single frequency. That is, the frequency response of the system in the absence of target analyte can be previously determined to be very low at a particular high frequency. Using this information, any response at a

25   particular frequency, will show the presence of the assay complex. That is, any response at a particular frequency is characteristic of the assay complex. Thus, it may only be necessary to use a single input high frequency, and any changes in frequency response is an indication that the analyte is present, and thus that the target sequence is present.

30   In addition, the use of AC techniques allows the significant reduction of background signals at any single frequency due to entities other than the analytes, i.e. "locking out" or "filtering" unwanted signals. That is, the frequency response of a charge carrier or redox active molecule in solution will be limited by its diffusion coefficient and charge transfer coefficient. Accordingly, at high frequencies, a charge carrier may not diffuse rapidly enough to transfer its charge to the electrode, and/or the charge transfer

35   kinetics may not be fast enough. This is particularly significant in embodiments that do not have good monolayers, i.e. have partial or insufficient monolayers, i.e. where the solvent is accessible to the electrode. As outlined above, in DC techniques, the presence of "holes" where the electrode is accessible to the solvent can result in solvent charge carriers "short circuiting" the system, i.e. the reach the electrode and generate background signal. However, using the present AC techniques, one or

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more frequencies can be chosen that prevent a frequency response of one or more charge carriers in solution, whether or not a monolayer is present. This is particularly significant since many biological fluids such as blood contain significant amounts of redox active molecules which can interfere with amperometric detection methods.

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In a preferred embodiment, measurements of the system are taken at at least two separate frequencies, with measurements at a plurality of frequencies being preferred. A plurality of frequencies includes a scan. For example, measuring the output signal, e.g., the AC current, at a low input frequency such as 1 - 20 Hz, and comparing the response to the output signal at high frequency such

10   as 10 - 100 kHz will show a frequency response difference between the presence and absence of the RAM. In a preferred embodiment, the frequency response is determined at at least two, preferably at least about five, and more preferably at least about ten frequencies.

After transmitting the input signal to initiate electron transfer, an output signal is received or detected.

15   The presence and magnitude of the output signal will depend on a number of factors, including the overpotential/amplitude of the input signal; the frequency of the input AC signal; the composition of the intervening medium; the DC offset; the environment of the system; the nature of the RAM; the solvent; and the type and concentration of salt. At a given input signal, the presence and magnitude of the output signal will depend in general on the presence or absence of the target analyte, the placement

20   and distance of the RAM from the surface of the monolayer and the character of the input signal. In some embodiments, it may be possible to distinguish between non-specific binding of materials and the formation of target specific assay complexes, on the basis of impedance.

In a preferred embodiment, the output signal comprises an AC current. As outlined above, the

25   magnitude of the output current will depend on a number of parameters. By varying these parameters, the system may be optimized in a number of ways.

In general, AC currents generated in the present invention range from about 1 femtoamp to about 1 milliamp, with currents from about 50 femtoamps to about 100 microamps being preferred, and from

30   about 1 picoamp to about 1 microamp being especially preferred.

In a preferred embodiment, the output signal is phase shifted in the AC component relative to the input signal. Without being bound by theory, it appears that the systems of the present invention may be sufficiently uniform to allow phase-shifting based detection. That is, the complex biomolecules of the

35   invention through which electron transfer occurs react to the AC input in a homogeneous manner, similar to standard electronic components, such that a phase shift can be determined. This may serve as the basis of detection between the presence and absence of the analyte and/or differences between the presence of target-specific assay complexes and non-specific binding of materials to the system components.

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The output signal is characteristic of the presence of the analyte; that is, the output signal is characteristic of the presence of the target-specific assay complex. In a preferred embodiment, the basis of the detection is a difference in the faradaic impedance of the system as a result of the formation of the assay complex. Of importance in the methods of the invention is that the faradaic

5   impedance between the RAM and the electrode may be signficantly different depending on whether the targets are specifically or non-specifically bound to the electrode.   •

Accordingly, the present invention further provides electronic devices or apparatus for the detection of analytes using the compositions of the invention. The apparatus includes a test chamber for receiving a

10   sample solution which has at least a first measuring or sample electrode, and a second measuring or counter electrode. Three electrode systems are also useful. The first and second measuring electrodes are in contact with a test sample receiving region, such that in the presence of a liquid test sample, the two electrodes may be in electrical contact.

15   In a preferred embodiment, the apparatus also includes detection electrodes comprising the compositions of the invention, including redox active complexes including binding ligands and redox active molecules, and a monolayer comprising conductive oligomers, such as are described herein.

The apparatus further comprises an AC voltage source electrically connected to the test chamber; that

20   is, to the measuring electrodes. Preferably, the AC voltage source is capable of delivering DC offset voltage as well.

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In a preferred embodiment, the apparatus further comprises a processor capable of comparing the input signal and the output signal. The processor is coupled to the electrodes and configured to receive

25   an output signal, and thus detect the presence of the target.

Thus, the compositions of the present invention may be used in a variety of research, clinical, quality control, or field testing settings.

30   In a preferred embodiment, viral and bacterial detection is done using the complexes of the invention. In this embodiment, binding ligands (for example antibodies or fragments thereof) are designed to detect targets (for example surface proteins) from a variety of bacteria and viruses. For example, current blood-screening techniques rely on the detection of anti-HIV antibodies. The methods disclosed herein allow for direct monitoring of circulating virus within a patient as an improved method of

35   assessing the efficacy of anti-viral therapies. Similarly, viruses associated with leukemia, HTLV-I and HTLV-II, may be detected in this way. Bacterial infections such as tuberculosis, clymidia and other sexually transmitted diseases, may also be detected. Similarly, the compositions of the invention find use as probes for toxic bacteria in the screening of water and food samples. Similarly, bioremediation strategies may be evaluated using the compositions of the invention.

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The present invention provides methods which can result in sensitive detection of target analytes. In a preferred embodiment, less than about 10^{12} molecules are detected, with less than about 10^{10} being preferred, less than 10^8 being particularly preferred, less than about 10^5 being especially preferred, and less than about 10^4 being most preferred.

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All references cited herein are incorporated by reference in their entireity.

CLAIMS

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We claim:

1. A composition comprising an electrode comprising:
    a) a self-assembled monolayer; and
15   b) a metal ion ligand covalently attached to said electrode via a conductive oligomer.

2. A composition according to claim 1 wherein said electrode comprises a plurality of different metal ion ligands.

20   3. A composition according to claim 1 wherein said metal ion ligand is phenanthroline.

4. A composition according to claim 1 wherein said conductive oligomer is selected from the group consisting of:

    i)

25

$$-\left(-Y-\left(-(B)_g-D-\right)_e\right)_n\left(-Y-\right)_m$$
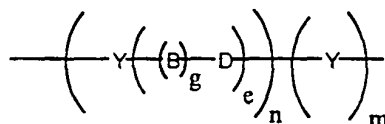
wherein
        Y is an aromatic group;
30   n is an integer from 1 to 50;
        g is either 1 or zero;
        e is an integer from zero to 10; and
   m is zero or 1;
        wherein when g is 1, B-D is a conjugated bond; and
35   wherein when g is zero, e is 1 and D is preferably carbonyl, or a heteroatom moiety, wherein
            the heteroatom is selected from oxygen, sulfur, nitrogen, silicon or phosphorus; or

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ii)

$$-\left(-C-G-C-\right)_n\left(-J-\right)_m-$$
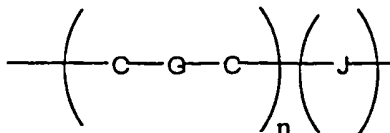
wherein

n is an integer from 1 to 50;

m is 0 or 1;

C is carbon;

J is carbonyl or a heteroatom moeity, wherein the heteroatom is selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur; and

G is a bond selected from alkane, alkene or acetylene, wherein if m = 0, at least one G is not alkane.

5. A method of detecting a metal ion comprising:

    a) applying a first input signal to an assay complex comprising:

        i) an electrode comprising:

            1) a self-assembled monolayer;

            2) a metal ion ligand covalently attached to said electrode via a conductive oligomer;

        ii) a metal ion;

    b) detecting a change in the faradaic impedance of the system as a result of the association of the metal ion with the metal ion ligand.

6. A method of detecting a non-nucleic acid target analyte in a sample comprising:

    a) applying a first input signal to an assay complex comprising:

        i) a redox active complex comprising:

            1) a redox active molecule;

            2) a binding ligand that will bind the target analyte; and

        ii) a target analyte;

    wherein at least one component of said assay complex is covalently attached to an electrode via conductive oligomer; and

    b) detecting a change in the faradaic impedance of the system as a result of the association of the redox active molecule with the target analyte, if present.

7. A method according to claim 6 wherein said electrode further comprises a self-assembled monolayer.

8. A method according to claim 6 wherein said input signal comprises an AC component.

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9. A method according to claim 8, wherein said input signal further comprises a DC component.

10. A method according to claim 6 wherein said redox active molecule is covalently attached to said electrode.

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11. A method according to claim 6 wherein said binding ligand is covalently attached to said electrode.

12. A method according to claim 6 wherein said redox active molecule is a transition metal complex.

10   13. A method according to claim 12 wherein said transition metal complex is ferrocene.

14. A method according to claim 6 wherein said redox active molecule is covalently attached to said binding ligand.

15   15. A method according to claim 6 wherein said detecting is by receiving an output signal characteristic of the presence of said analyte.

16. A method according to claim 15 wherein said output signal comprises a current.

20   17. A method according to claim 6 wherein said conductive oligomer is selected from the group consisting of:

i)

$$-\left(-Y-\left(-(B)_g-D-\right)_e\right)_n\left(-Y-\right)_m$$
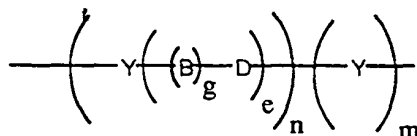
25   wherein

Y is an aromatic group;
n is an integer from 1 to 50;
g is either 1 or zero;
e is an integer from zero to 10;and
30   m is zero or 1;
wherein when g is 1, B-D is a conjugated bond; and
wherein when g is zero, e is 1 and D is preferably carbonyl, or a heteroatom moiety, wherein the heteroatom is selected from oxygen, sulfur, nitrogen, silicon or phosphorus; or

ii)
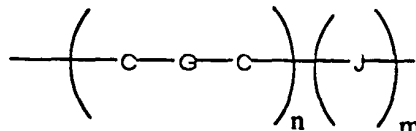
35

$$-\left(-C-G-C-\right)_n\left(-J-\right)_m$$

wherein

n is an integer from 1 to 50;
m is 0 or 1;

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C is carbon;

J is carbonyl or a heteroatom moeity, wherein the heteroatom is selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur; and

G is a bond selected from alkane, alkene or acetylene, wherein if m = 0, at least one G is not

5   alkane.

18. An apparatus for the detection of a non-nucleic acid target analyte in a test sample, comprising:

a) a test chamber comprising at least a first and a second measuring electrode, wherein said first measuring electrode comprises:

10   i) a self-assembled monolayer;

ii) a binding ligand covalently attached to said electrode via conductive oligomer;

b) a voltage source electrically connected to said test chamber.

19. An apparatus according to claim 18, further comprising a processor.

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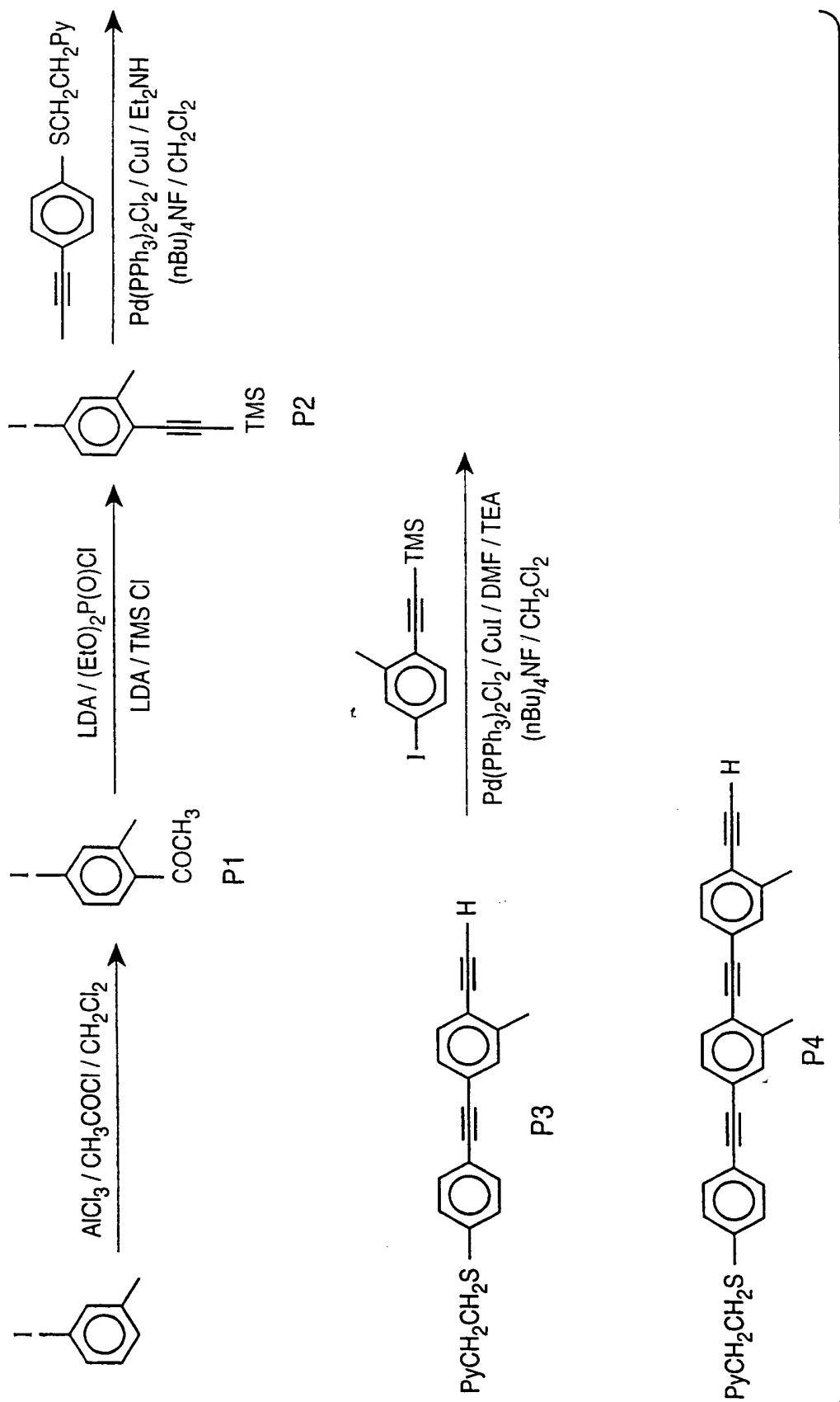
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FIG._1

FIG._2

FIG._3A

FIG._3B

FIG._3

FIG._4

FIG._5

FIG._6A

FIG._6B

AC CURRENT / 1e-7A
POTENTIAL / V

FIG._6C

AC CURRENT / 1e-7A
POTENTIAL / V
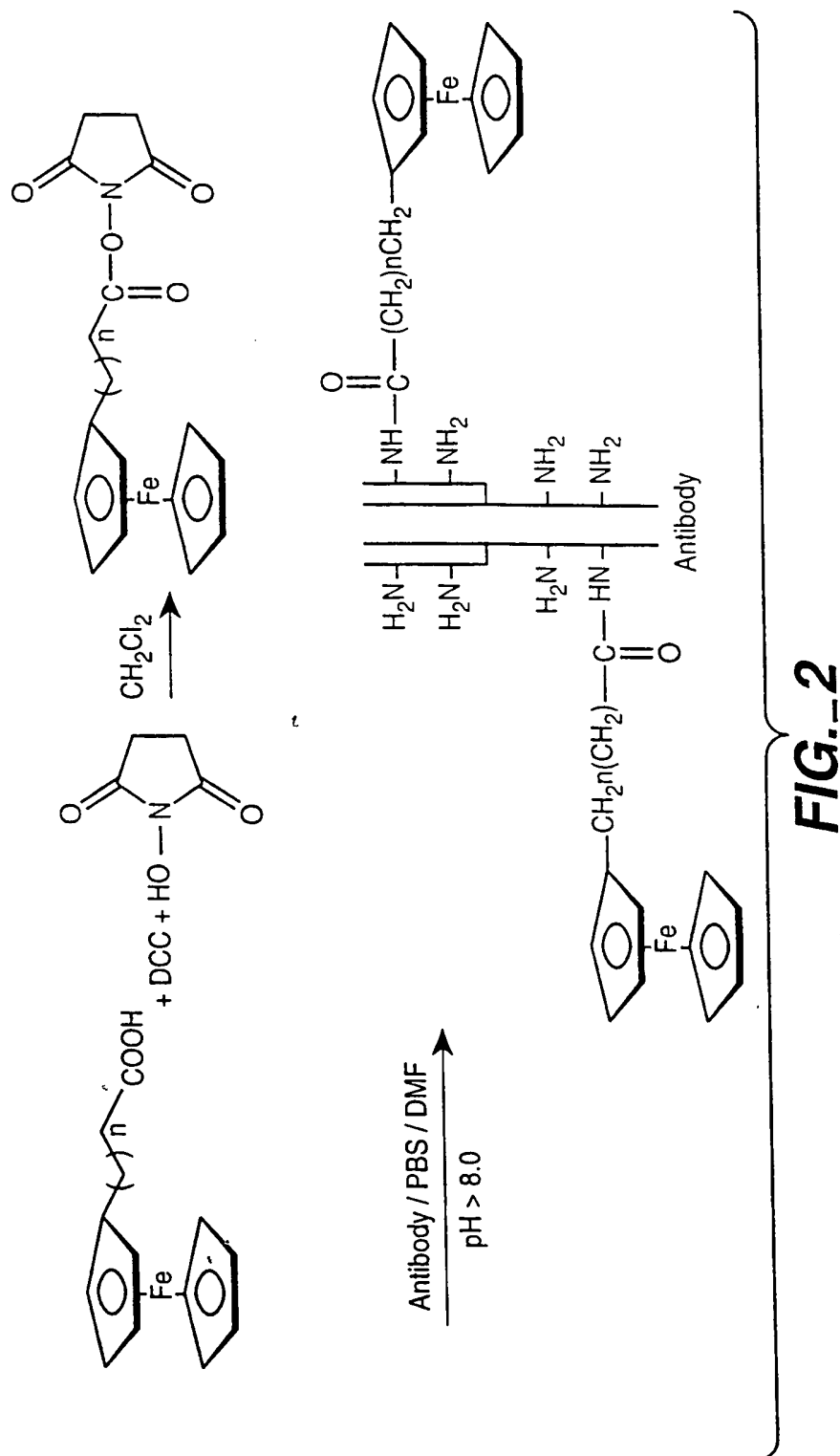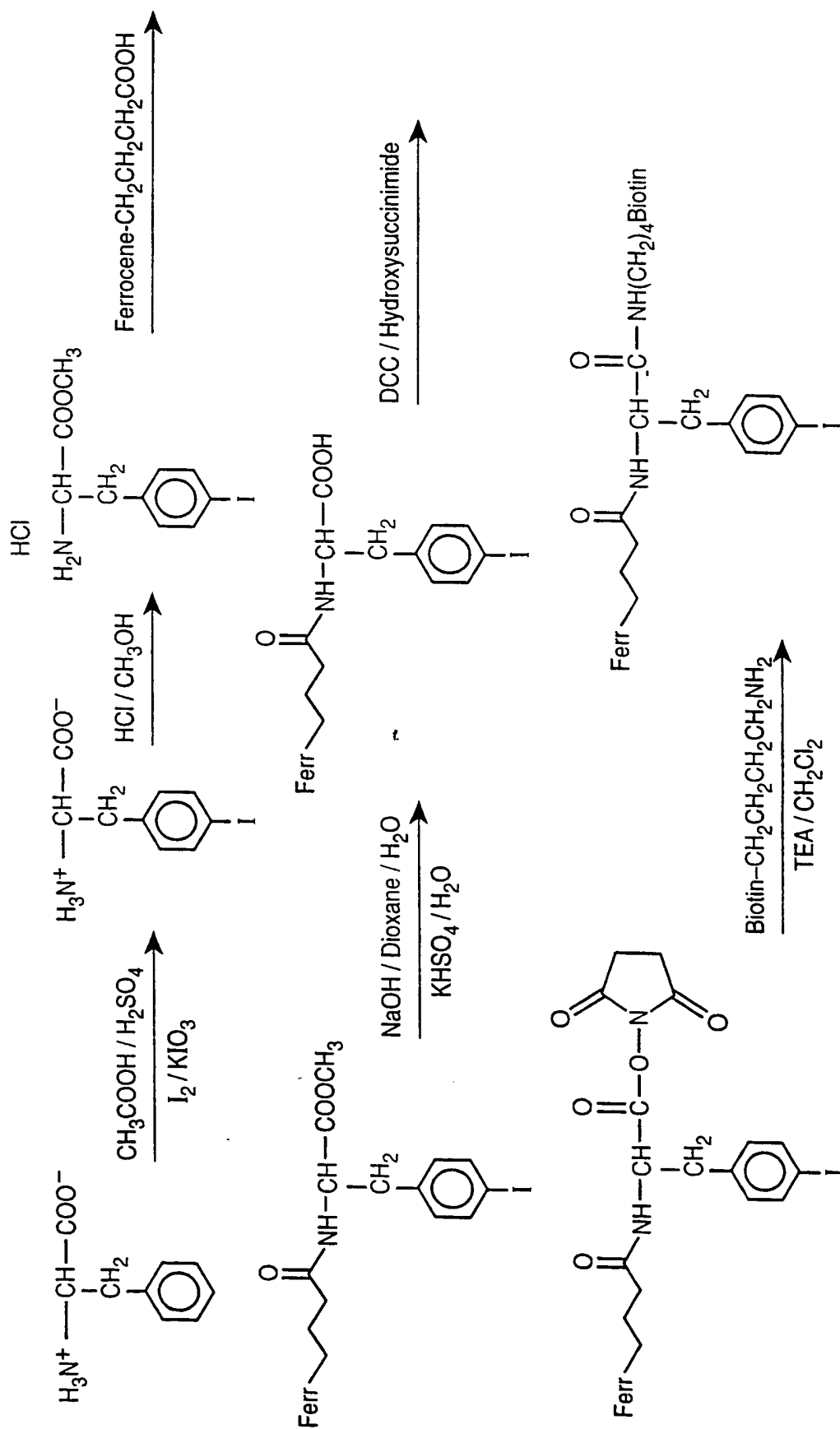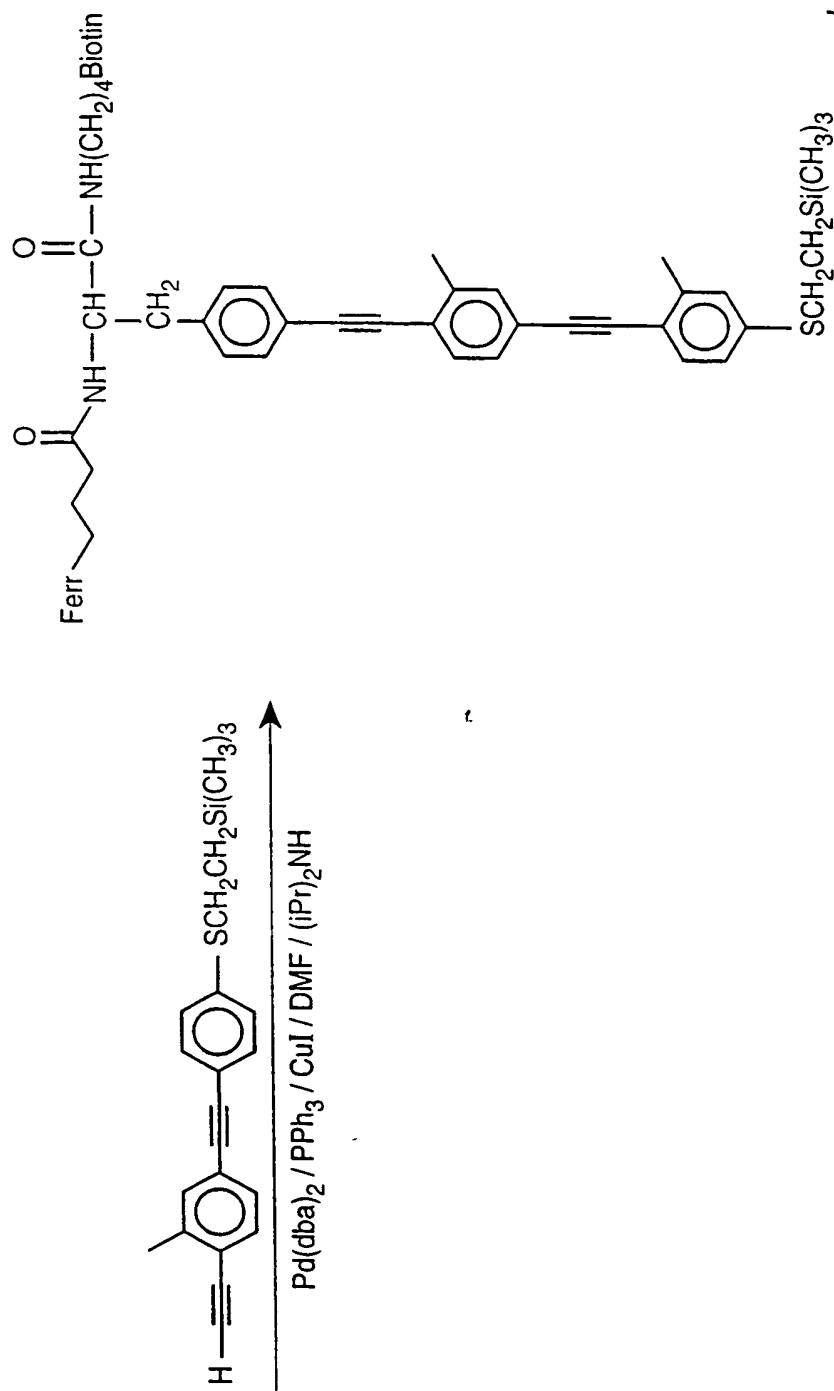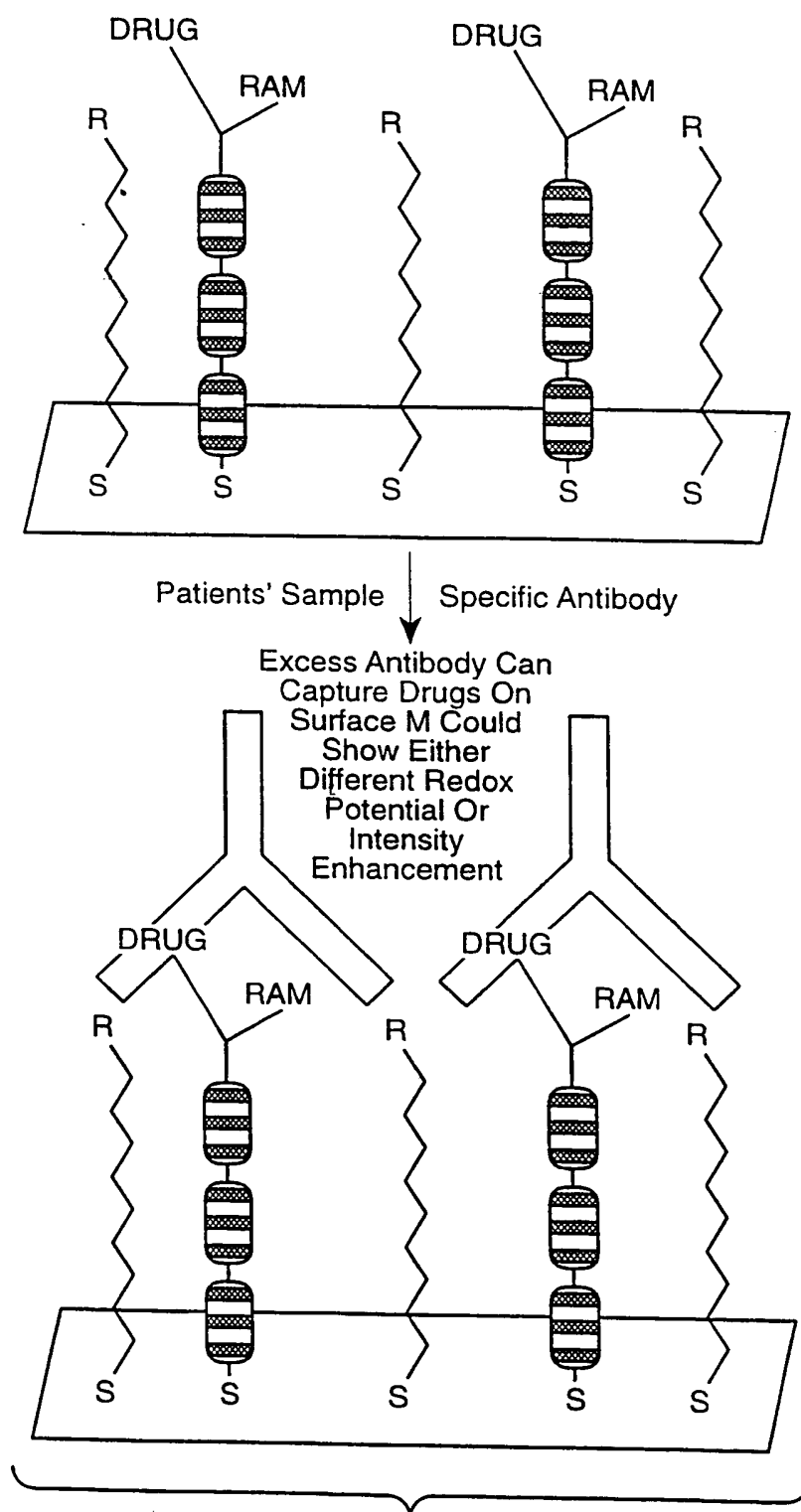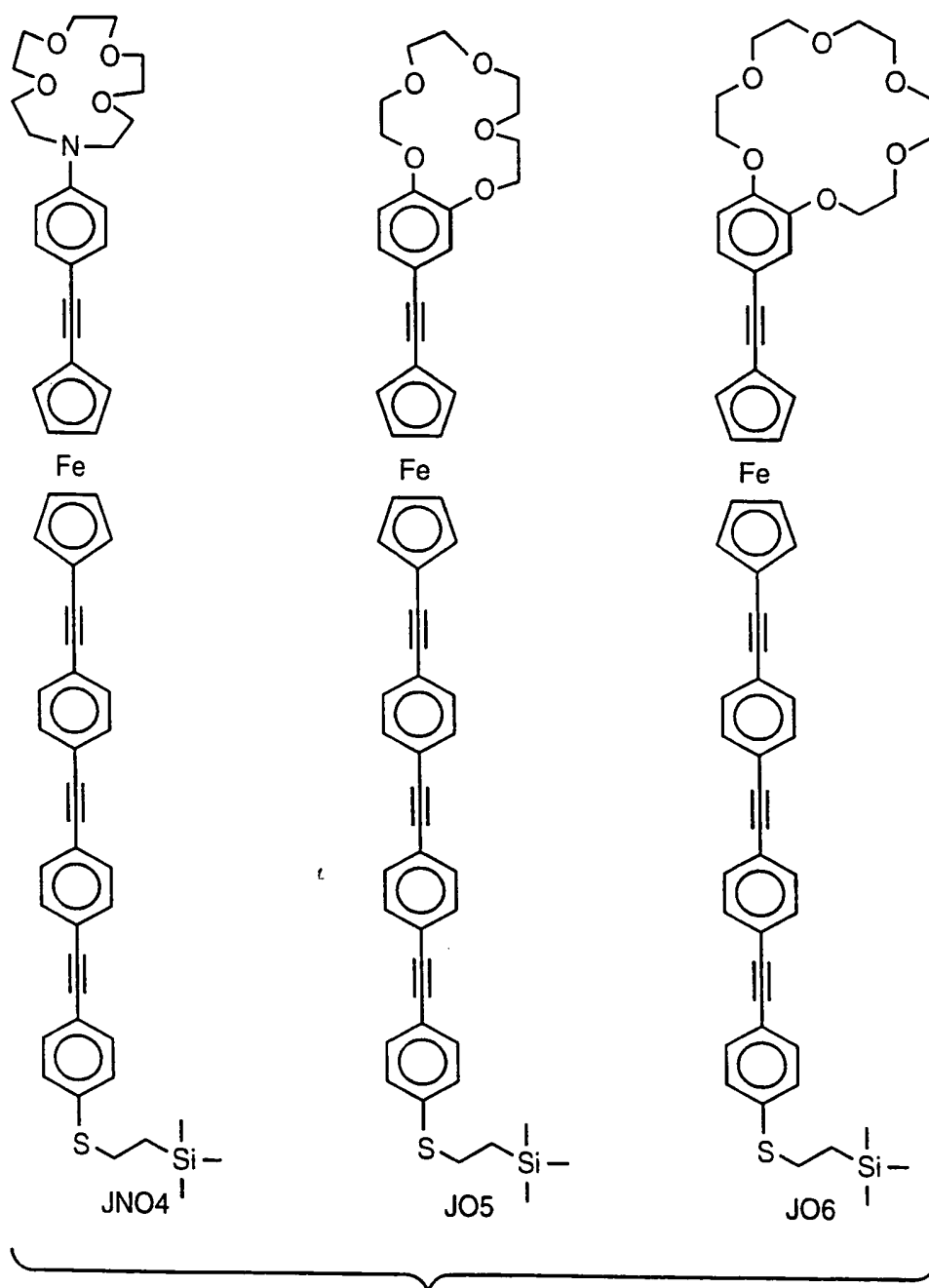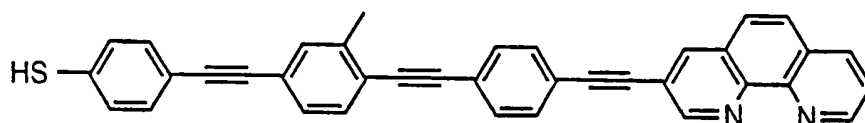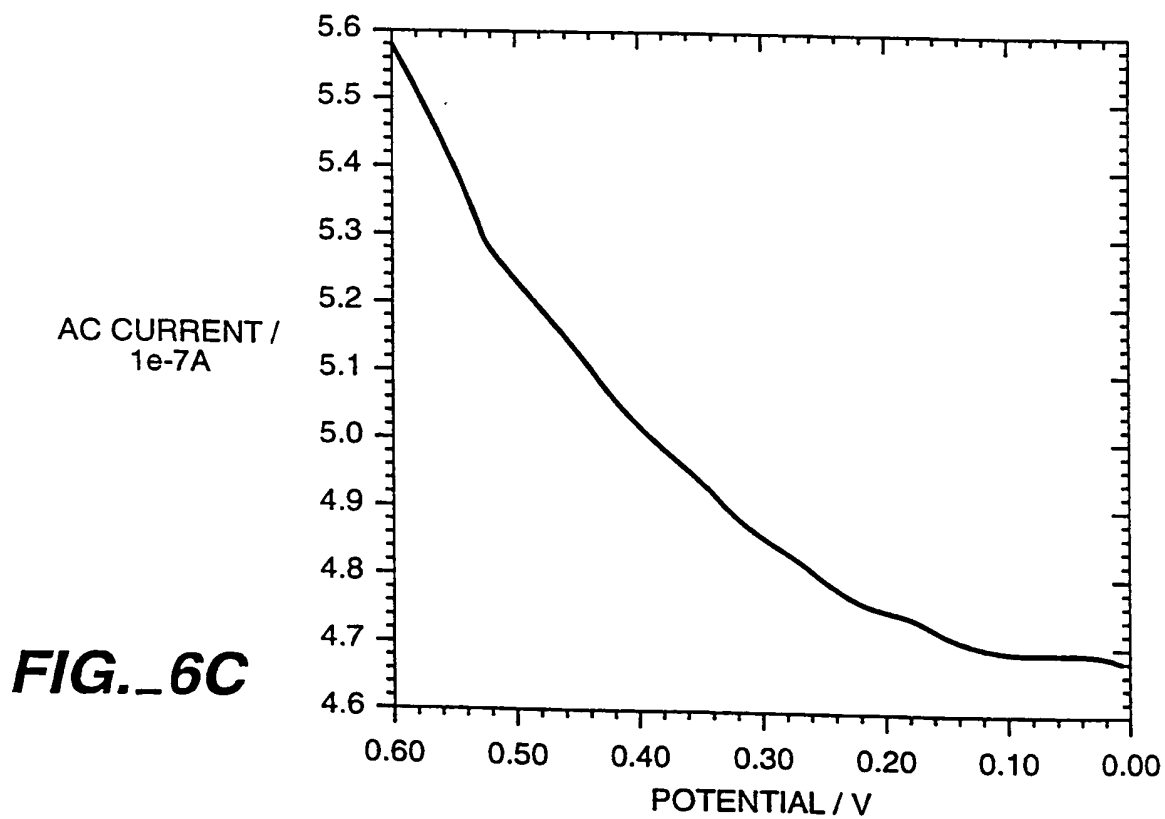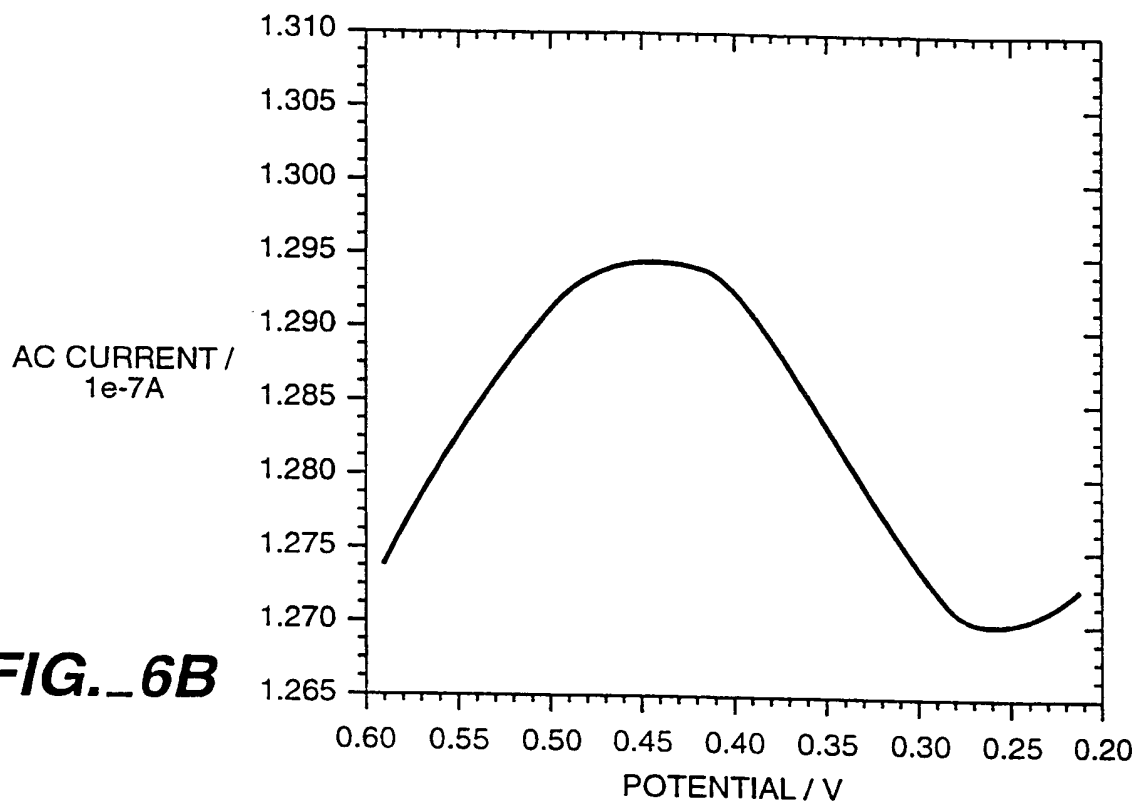
INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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US 5108573	A	28-04-1992	NONE		

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document. with indication.where appropriate. of the relevant passages	Relevant to claim No.
A	W. M. ALBERS ET AL: "Design of novel molecular wires for realizing long-distance electron transfer." BIOELECTROCHEMISTRY AND BIOENERGETICS , vol. 42, 1997, pages 25-33, XP002077970 see the whole document ---	1,5,6,18
A	R. P. HSUNG ET AL: "Synthesis and characterization of unsymmetric ferrocene-terminated phenylethynyl oligomers." ORGANOMETALLICS, vol. 14, no. 10, 1995, pages 4808-4815, XP002077968 see the whole document -----	1

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   G01N27/327   G01N33/543   G01N33/84   G01N27/333

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and. where practical. search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 571 568 A (RIBI HANS O ET AL) 5 November 1996 see abstract see column 2, line 55 – column 3, line 2 ---	1,4-6,18
X	AIZAWA M ET AL: "INTEGRATED MOLECULAR SYSTEMS FOR BIOSENSORS*" SENSORS AND ACTUATORS B, vol. B24, no. 1/03, PART 01, March 1995, pages 1-5, XP000521315 , see the whole document ---	1,5,6,18
A	US 5 108 573 A (RUBINSTEIN ISRAEL ET AL) 28 April 1992 see abstract --- -/--	1,5,6,18

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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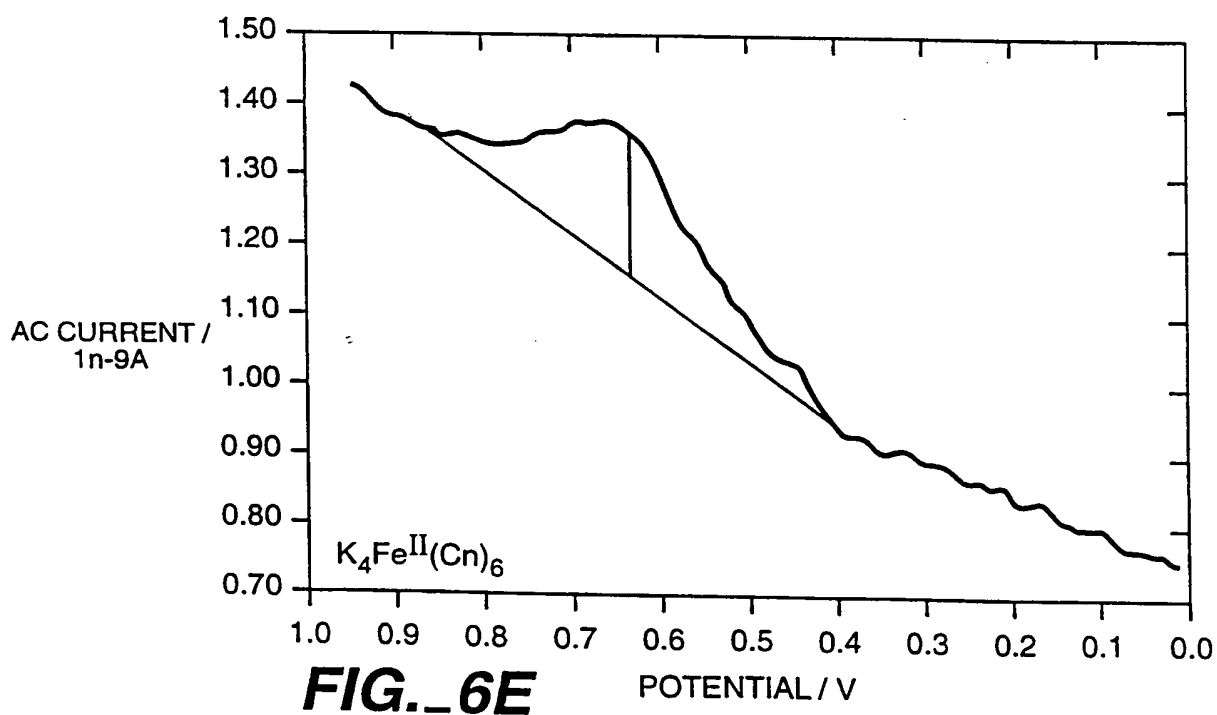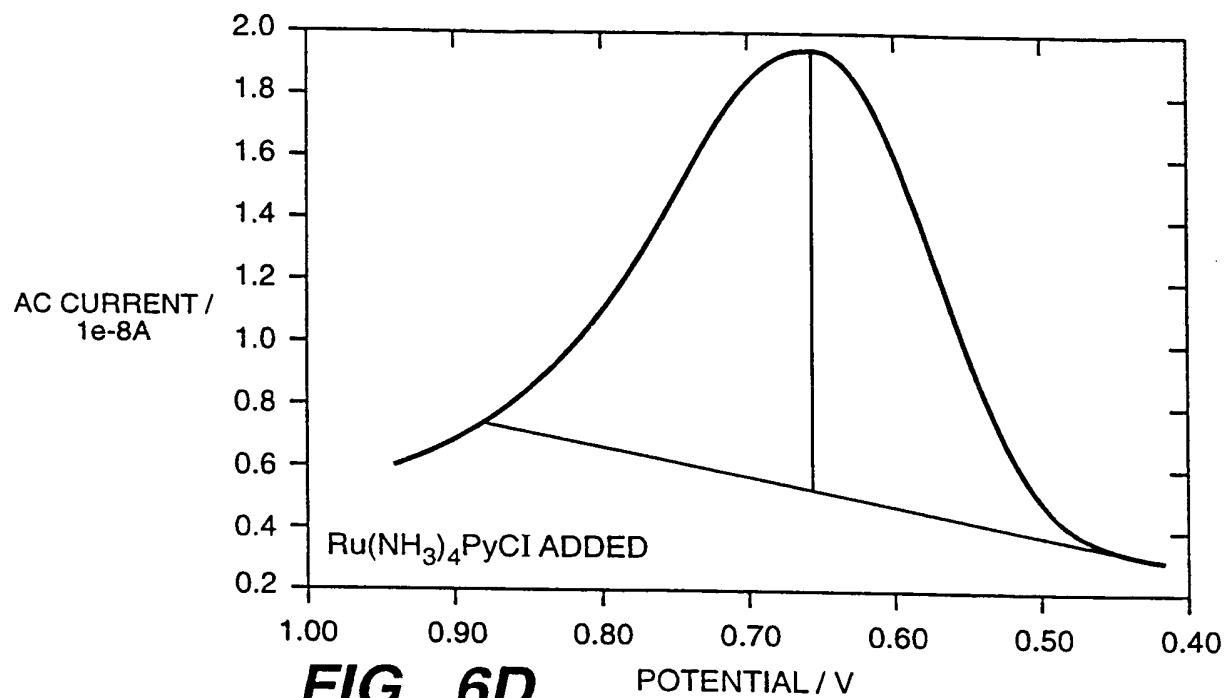| Date of the actual completion of the international search	Date of mailing of the international search report
1 October 1998	09/10/1998

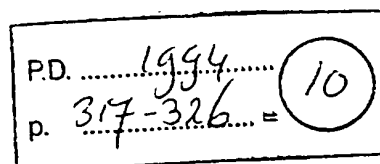| Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreno, C

Form PCT/ISA/210 (second sheet) (July 1992)

FIG._6D

FIG._6E

XP-002106965

Metalloorganic labels for DNA sequencing and mapping

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Abstract. – The sequencing and mapping of DNA and the technologies to accomplish these tasks are rapidly evolving. Sequence information can give insite into the location and function of genes, provide an understanding of the relationship between variations in sequence and different genetic diseases, and serve as a basis for the prediction, treatment and cure of those disease conditions. In pursuit of these capabilities the Human Genome Program has proposed sequencing the entire human genome over the next 15 years. The work presented here represents our efforts to advance the technology for faster and more automated sequencing procedures. Focusing on metal-centered organic compounds and a variety of detection methods, several strategies for labeling and detecting DNA sequencing reaction products are presented. The synthesis and testing of a variety of compounds and approaches are discussed including mass labels using tin, iron and lanthanide elements, electrochemically detectable ferrocene derivatives, and luminescent complexes of given lanthanides. The results of this work are discussed and future directions presented.

The Human Genome Program is an ambitious effort with the goal of initially constructing a high resolution genetic map and later locating and sequencing the estimated 100,000 genes found within the 23 human chromosome pairs. Some of the objectives of the program, jointly administered by the Department of Energy and the National Institutes of Health, are to complete a physical map of the chromosomes by 1995 and completely sequence the genome within 15 years [1]. If this task is to be accomplished in this period of time, faster, less expensive, and less labor intensive methods of DNA sequencing and mapping must be developed. Conventional sequencing and mapping techniques generally require that fragments of DNA be separated by size or length of chain, usually by electrophoresis, and then the positions of the fragments must be identified. One method of achieving greater speed in this process is to employ multiple, unique labels in a process called multiplex analysis [2]. However, since DNA sequence and mapping analysis is intimately tied to the separation of DNA fragments by chain length, multiplex analysis requires that the labels do not differentially or unpredictably alter the electrophoretic mobilities of the DNA samples under investigation.

Traditional methods of DNA sequence and hybridization analysis have relied on the introduction of radioactiv phosphorous or sulfur isotopes into the DNA. DNA fragments, either chemically or enzymatically generated, are separated by chain length using slab gel electrophoresis, and the presence of the label is detected by autoradiography using X-ray film. The use of fluorescent organic compounds as labels for DNA offers a number of advantages over radiolabels, and the use of these compounds for sequence analysis has increased dramatically in recent years [3, 4, 5] to where they are currently the most commonly used labels for both DNA sequencing and mapping. An instrument to detect fluorescent dye-labeled DNA fragments following their electrophoretic separation has been developed and is now commercially available [6]. Fluorescent reporters are preferable to radioactive labels since they can be detected in real-time at comparable levels. They also lack the hazards associated with radiation and the associated

problems of waste handling and disposal. Fluorescent labels will also permit the multiplexing of samples, although the simultaneous analysis of multiple fluorescent labels is difficult. The organic dyes which have been used to date have broad emission maxima which seriously limits the number of simultaneously employed dyes. Also, when high levels of sensitivity are required, distinguishing individual fluorophores is quite difficult. Additionally, the effects of the structure of different dyes upon the mobility of the fragments to which they are attached must be minimal, or at least predictable, further limiting the number of usable compounds.

We are developing alternative labels and detection systems which have the strengths of fluorophores while lacking many of their shortcomings. Based on the use of metallic elements and their compounds, each of our labeling approaches can be readily utilized in multiplex analyses. Their detection is accomplished by one of three methods, mass spectrometry, emission spectroscopy, or electrochemical amperometry, each having the required sensitivity for our purpose. In addition, these compounds meet those major requirements of any labeling scheme whereby the labels are stable to the conditions of the sequencing reactions and their presence does not interfere with hybridization and/or replication reactions of the DNA. When employed in multiplex analyses, these complexes of metallic elements are also kinetically inert to exchange between metal sites.

Detection methods

The metallic elements we employ are not natural constituents of DNA and each isotope of these elements can serve as a unique label. Resonance ionization spectroscopy-mass spectrometry (RIS/MS) is a highly specific, very sensitive analytical technique that affords selectivity by element and isotope and thus is ideal when dealing with discrete mass labels. RIS/MS uses an ion beam (sputter initiated RIS, SIRIS) or a laser beam (laser atomization RIS, LARIS) to create atoms of the substance to be analyzed. Samples of electrophoretically separated DNA fragments, along with the matrix containing them, are converted to gas phase atoms by one of the ablative methods mentioned. A very high degree of elemental specificity is then gained by employing narrow bandwidth lasers tuned to ionize only the specific element of interest. High sensitivity is attained by virtue of the high pulse energies of the ionizing laser beams that essentially ionize all of the selected atoms in the laser beam path. The ions are accelerated and introduced into a mass analyzer (either a magnetic sector mass analyzer or a time-of-flight instrument) for discrimination and identification.

Associating the detected isotope with one of the four bases of DNA in a sequencing reaction for which it was used then permits the positive identification of the sample fragment. Four labels are required for DNA sequencing if analysis of the reactions is carried out simultaneously. Elements that are readily ionized by the laser based RIS method and have four or more stable isotopes are potential candidates for DNA sequencing labels. The details of the RIS/MS process as applied to DNA sequencing and mapping have been reviewed by Jacobson and Arlinghaus [7]. In a recent review of mass spectrometric approaches to DNA sequencing [8], mass-labeling DNA with subsequent detection of gel-separated fragments by RIS/MS was judged as the fastest of all such analytical approaches.

A number of metal complexes have excited states with emission lifetimes which are long compared to those of the organic dyes currently employed for DNA sequencing or mapping. A different detection approach, utilizing pulsed-excitation and time-gated emission analysis, can take advantage of these long lifetimes for enhanced background or impurity fluorescence rejection. Europium chelates are examples of compounds that possess these characteristics and which are of interest to us for labeling DNA. Their use in DNA hybridization applications with detection by time-resolved emission spectroscopy has been reported [9].

Electrochemical detection devices have been successfully employed as analytical tools for some time. Amperometry of electroactive species has demonstrated sensitivity levels in the femtomole and attomole range. For these reasons we have become interested in electroactive organometallic compounds for use as labels for DNA fragments. Specifically we have chosen ferrocene and a number of its derivatives as viable alternatives to other DNA reporters currently in use. To better insure the success of this approach we intend to combine amperometric detection and capillary gel electrophoresis into a powerful integrated analytical system.

Current trends in the evolution of gel electrophoresis have led to increasing use of capillary gel electrophoresis [10, 11, 12] or very thin slab gel electrophoresis [13] for separations. These gel formats dissipate heat better than conventional slab gels allowing the use of higher voltage for increased resolution and greater speed. When using capillary systems, increased analytical throughput is achieved by simultaneously running a number of capillaries with a single excitation source-detector combination scanning over the array [12]. Multiple lanes of a thin slab gel format can also be run simultaneously with a similar scanning detection scheme [13].

Amperometry of capillary separated analytes began with the open tubular chromatography work of

Jorgenson [14], and was subsequently extended to capillary zone electrophoresis (CZE) analyses, most notably by Ewing and co-workers [15]. To acccomplish amperometric analyses of electroactive substances undergoing capillary electrophoretic separations, it is necessary to transport those analytes to an electrochemical cell that is outside (off-column) of the high voltage separation field. This is necessary to limit the interference of the high voltage separation field with the applied potential of the electrochemical analysis cell. When separations are performed using CZE, the electroendosmotic flow of solution within the capillary aids in the separation of analytes and provides a means for their transport from the column. CZE, however, can not provide the needed size discrimination for DNA due to the similarities in charge-mass ratios of DNA fragments larger than 20 bases. Instead, capillary gel electrophoresis (CGE) must be used since it introduces the necessary frictional component required to successfully effect DNA fragment size discrimination. When separating DNA by CGE the normal polarity of the separation field is reversed since DNA carries a large negative charge and migrates towards the anode. It is also necessary to suppress electroendosmosis when performing CGE since the electrical bilayer that typically forms in the capillary in response to the applied high voltage field induces a bulk flow of solution towards the cathode. This flow would seriously interfere with the electrophoretic separation of DNA which is moving in the opposite direction. Since electroendosmotic flow must be suppressed, alternative methods, such as the use of the sheath flow cúvette as demonstrated by Dovichi and coworkers [11], are required to accomplish the transport of labeled DNA fragments to the off-column analytical cell.

The analytical cell required for the detection of a single electroactive species would be quite uncomplicated. The simplest cell would consist of two electrodes, a working electrode with a fixed potential (slightly exceeding the half-wave potential of the electroactive label) relative to the second, or reference electrode. DNA fragments, tagged with electroactive ferrocene derivatives, would be directed to this cell following their separation. A fixed potential would be applied to the cell to oxidize the electroactive reporter at the working electrode while the measurable current resulting from the induced oxidation reaction would serve as the means to detect and identify the DNA fragment associated with the label. Design features for an analytical cell suitable for integration into a capillary gel electrophoresis system are currently under consideration. Efforts are being directed towards the design of both a simple, single working electrode system, and a multiple working electrode system capable of detecting several electroactive species concurrently.

Synthesis of labels and attachment to DNA

Ferrocene labels

Ferrocene and its derivatives have been developed as DNA reporters from the standpoint of two disparate approaches to their detection, mass signature and electroactivity. In the mass-labeling approach, stable isotopes of iron serve as the identifiers of specific DNAs. The isotopes are incorporated in ferrocene carriers which are attached to the DNA primers used in the sequencing reactions and are thus present in each fragment of DNA that arises from those reactions. For electrochemical detection, the Fe (II)/Fe (III) couple of ferrocene is the specific label, and the inductive effects of different substituents on the cyclopentadienyl rings change the half-wave potential of the redox couple, allowing different labels to be generated with only minor changes in the size or chemical reactivity of the label.

Of the four stable isotopes of iron two, ^{56}Fe and ^{57}Fe, have been incorporated into ferrocene to serve as DNA labels [16]. Synthesized as N-hydroxysuccinimide (NHS) esters, the reporters were subsequently attached to oligonucleotides (see Fig. 1) and used to prime the Sanger sequencing reactions and polymerase chain reaction (PCR) amplifications of DNA. To provide a reactive site for the NHS ester, a hexylamine group was added to the oligomer in the final step of its synthesis. The NHS group, which is quite reactive with primary amines, was reacted with the 5' terminal amine of the oligomer, resulting in the incorporation of the reporter group via an amide linkage.

The NHS ester of ^{56}Fe-ferrocene was synthesized from commercially available (91.7%, ^{56}Fe) ferrocene carboxylic acid using a modification of the method of Anderson et al. [17]. In that procedure (Fig. 1), one molar equivalent of ferrocene carboxylic acid was reacted, under argon, with two equivalents of NHS using the coupling reagent DCC in anhydrous THF. The product was chromatographed on silica gel to obtain N-hydroxysuccinimidyl-ferrocene carboxylate in 80% yield.

The synthesis of the NHS ester of ^{57}Fe-ferrocene involved converting ^{57}Fe$_2$O$_3$, obtained from the Oak Ridge Isotope Distribution Office and enriched to 67.9% ^{57}Fe, to ferrocene using a modification of the procedure of Ekemark and Skagius [18]. Iron oxide was dissolved in hydrochloric acid, and then quantitatively electroplated onto a platinum gauze electrode from a slightly acidic oxalate solution. Under an argon atmosphere, and using strictly anaerobic reagents and solvents, the iron metal was oxidized to iron (II) chloride by the use of dry hydrogen chloride in ethanol. To accomplish this, the lectrode was covered with anhydrous, oxygen-free
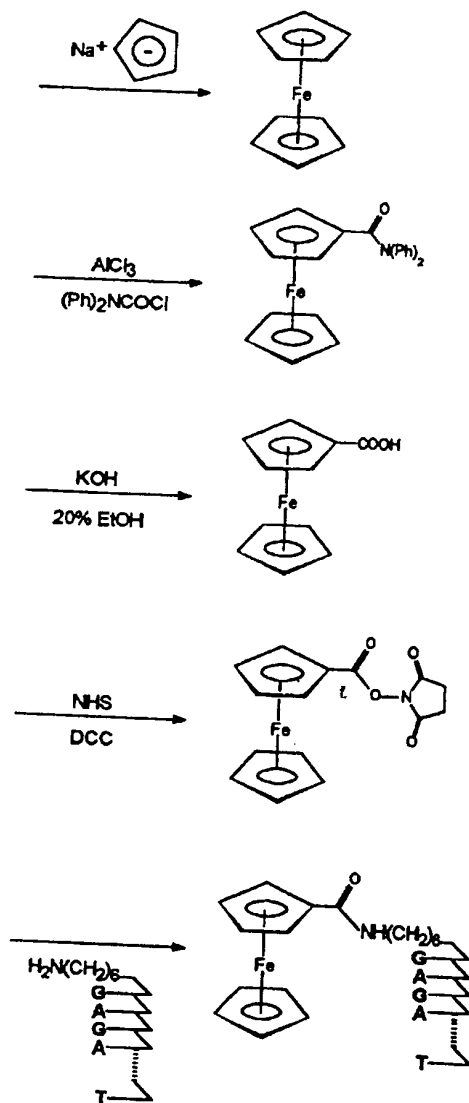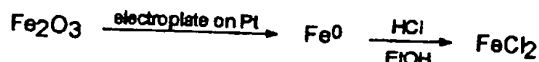
$$Fe_2O_3 \xrightarrow{\text{electroplate on Pt}} Fe^0 \xrightarrow[\text{EtOH}]{\text{HCl}} FeCl_2$$

Figure 1. – Synthesis of ferrocene from Fe_2O_3 and its attachment to DNA oligonucleotides.

ethanol to which acetyl chloride was added by syringe. Following solvent removal by vacuum, the iron (II) chloride was dissolved in anhydrous, oxygen-free THF. Sodium cyclopentadienylide was added and allowed to

react to form [57]Fe-ferrocene. The product was eluted from silica with THF, the solvent was removed, and following sublimation an 84% yield of [57]Fe-ferrocene was realized based on starting oxide.

Conversion of the [57]Fe-ferrocene to [57]Fe-ferrocene carboxylic acid was accomplished using the procedure of Little and Eisenthal [19]. [57]Fe-enriched ferrocene was reacted with $AlCl_3$ and diphenylcarbamyl chloride in 1,2-dichloroethane. The product, N,N-diphenyl ferrocenecarboxamide, was reacted with 20% ethanolic KOH and the acidified product extracted several times with ether. The combined ether extracts where dried, evaporated in vacuo, and chromatographed over silica gel to realize a 39-43% yield of [57]Fe-enriched ferrocene carboxylic acid. As previously described, the procedure of Anderson et al. [17], was used to convert the ferrocene carboxylic acid to [57]Fe-enriched N-hydroxysuccinimidyl ferrocene carboxylate. Labeled oligonucleotides were prepared with both normal isotopic composition NHS ferrocene carboxylate and [57]Fe-enriched NHS ferrocene carboxylate by reaction with a 5'-terminal hexylamine group attached to the oligo. The presence of the label was confirmed by routine diagnostic procedures using onlin , HPLC-integrated UV and electrochemical detection.

Triethylstannylpropanoic acid labels

Considerable effort has been directed towards the development of tin isotopes as labels for DNA. The selection of tin as a likely candidate for mass labeling DNA was based upon a number of criteria. As shown by our own analyses [20], endogenous tin levels in a number of the reagents and materials used when working with, and manipulating DNA, exhibited low (ppb) levels of the element, making it favorable for analysis by RIS/MS. In addition, tin forms stable organometallic compounds and has ten isotopes, eight of which are commercially available [21]. The organotin compound triethylstannylpropanoic acid (TESPA) was selected as a likely carrier molecule for tin isotopes and its NHS ester N-hydroxysuccinimidyl triethylstannylpropanoate (NHS-TESPA) could be readily reacted with an oligomer's 5'-terminal amine for the covalent attachment of the carrier. Adaptations of the synthetic schemes of Van der Kerk et al. [22-25] and Anderson et al. [17], resulted in a seven-step synthesis (Fig. 2, path 1) for the production of NHS-TESPA, with a 20-25% overall yield from tin oxide [26]. Subsequent efforts to improve the synthesis of this compound resulted in a five-step scheme (Fig. 2, path 2) that relied on one less chromatographic procedure, avoided a troublesome side reaction, and increased the overall yield by 60% [21].
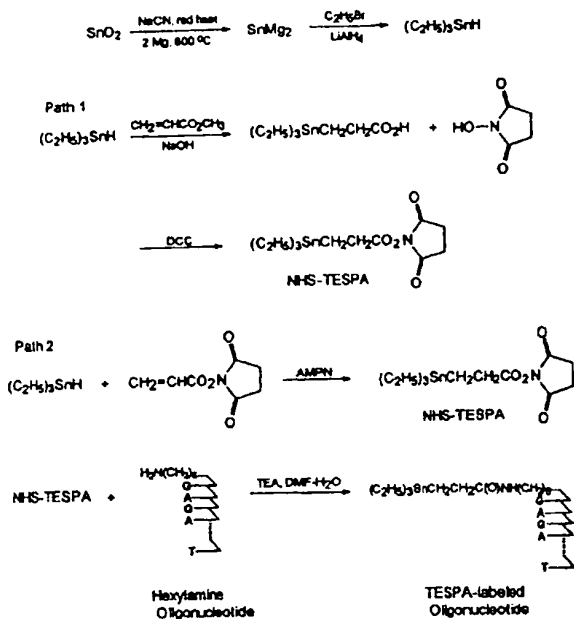
320

Figure 2. – Synthesis of TESPA from SnO_2 and its attachment to DNA oligomers. Path 1 represents our initial synthetic scheme involving adaptations from standard literature methods. Path 2 depicts an improved scheme for TESPA's synthesis involving two fewer synthetic steps.

In our initial synthetic scheme (Fig. 2, path 1) [26], tin oxide (SnO_2), enriched in a given isotope, was reduced with sodium cyanide (red heat, 94%) and then alloyed with magnesium (800°C, 97%). Reaction of the Mg_2Sn (finely ground) with bromoethane (sealed tube, 160°C, in cyclohexane, for 18 h) gave bromotriethylstannane at >85% purity by gas chromatography. The bromotriethylstannane was reduced quantitatively by reflux with lithium tetrahydridoaluminate (in ether, 6 h) to yield triethylstannane. Addition of triethylstannane to excess methyl acrylate, then product isolation by chromatography gave methyl 3-triethylstannylpropanoate at a 50% yield based on bromotriethylstannane. The methyl ester was base hydrolyzed with NaOH to give 3-triethylstannylpropanoic acid at 78% yield. This compound was then coupled with N-hydroxysuccinimide using dicyclohexylcarbodiimide with product isolation accomplished by chromatography on silica gel. This final product, NHS-TESPA, obtained at 76% yield, was later attached (Fig. 2) through an amide linkage to oligodeoxynucleotide primers, and as such provided the unique mass signatures for DNA fragments arising from reactions (Sanger sequencing and PCR) so primed.

The departure of the improved TESPA synthetic scheme (Fig. 2, path 2) from our original synthesis came after the production of triethylstannane. This product was reacted

with an equimolar amount of N-acryloxysuccinimide under argon in the presence of 1 mole percent of the free radical initiator 2,2'-azobis(2-methylpropionitrile) (AMPN) to yield NHS-TESPA. This product was isolated by silica gel chromatography at a yield of 51% from bromotriethylstannane. This synthetic route resulted in an overall final product yield of 40%, in two fewer synthetic steps, and only one chromatographic step. We also avoided the formation of an unwanted polymerization by-product, 3-triethylstannylmethyl glutaric acid, present in the fifth reaction step of the original synthetic procedure.

Lanthanide ion labels

Compounds of lanthanide elements have two properties which make them attractive as labels for DNA sequencing and mapping. There are 50 stable isotopes of elements in the lanthanide series (elements with atomic number 57 to 71) having an abundance of 0.1% or greater. When bound by a ligand system which attaches the Ln (III) ion to DNA, the electrophoretic mobility of all the lanthanide elements should be practically identical, creating an attractive system for multiplex analysis with mass labels. Moreover, the lanthanide ions Sm (III), Eu (III), Tb (III), and Dy (III) have luminescent excited states in fluid solution [27]. The excited states of complexes of lanthanide ions are long-lived, metal centered *f-f* states. The bandwidths are quite narrow, even at room temperature, which should allow multiple probes to be detected simultaneously with little overlap of emission. The relatively long lifetimes of the Ln (III) complexes allow the use of pulsed excitation and time-gated detection and thus virtually eliminate background signals from scattering processes and impurity fluorescence.

A derivative of the macrocyclic chelating agent, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) has been used to attach lanthanide ions to oligonucleotides. Lanthanide (III) ions form complexes with DOTA that are both thermodynamically and kinetically stable, with stability constants in the range 10^{27} to 10^{29} [28, 29]. Sequencing will be performed at neutral or higher pH where dissociation of the complexes is expected to be negligible. Meares and coworkers [30, 31] have prepared a *p*-nitrobenzyl derivative of DOTA so that radioisotopes of metal ions could be attached to protein molecules for applications such as tumor imaging. Reduction of the nitro group of the substituted DOTA ligand to an amine provides the functionality for attachment to DNA using reactions developed for attachment of fluorescent probes.

The *f-f* transitions of the lanthanide elements have very low oscillator strengths, and the reported sensitivity with direct *f-f* excitation for Eu (III) (and Tb (III)) complexes

with ligands such as DOTA appears to be lower than desired [27]. The emission intensity at fixed excitation intensity can be greatly enhanced if the excitation is ligand localized to a conjugated π-system (high extinction coefficient) having an energetic triplet state. Energy transfer from a ligand-based state to the emitting state of a lanthanide ion has been demonstrated to occur with high efficiency. Naphthalene is one group which has a lowest triplet energy state appropriate to transfer energy to Sm, Eu, Tb, or Dy. Excitation into naphthalene-based orbitals occurs with an absorption coefficient which is approximately three to five orders of magnitude larger than direct excitation into an *f-f* orbital. The organic moiety thus acts as an antenna to efficiently direct excitation energy to the Ln (III) ion and allows the use of a single excitation wavelength for all Ln (III) ions.

A high-yield, general synthetic route (see Fig. 3) was developed for the synthesis of derivatives of DOTA with an aryl substituent at one of the ethylene carbon atoms [32]. Benzyl and 2-naphthylmethyl derivatives have been prepared by this pathway. The methyl ester of the aryl substituted alanine is the starting material for the synthesis of substituted DOTA ligands. Tosyl protection of the amine followed by reaction with ethylenediamine, further protection, and reduction yield the tosyl protected diethylenetriamine with an aryl substituent. Ring formation was accomplished by treatment with cesium carbonate and 2,2'-(tosylimino)bisethyl ditosylate (Richman-Atkins conditions). Deprotection was accomplished by reduction with lithium tetrahydroaluminate and the acetate arms were attached by treatment with the *t*-butylester of bromoacetic acid. Acid hydrolysis of the *t*-butyl groups yielded the tetraacetic acid of the aryl substituted tetraazacyclododecane.

Nitration of a benzyl substituent (see Fig. 4) provided a point of attachment of the ligand to DNA. Nitration of the phenyl group was accomplished at the tetraaza-cyclododecane intermediate [32]. The acetate arms were added to the nitro-substituted macrocycle as previously outlined in Figure 3. Our procedure for preparation of *p*-nitrobenzyl-DOTA is a considerable improvement over the previously reported procedures [30, 31, 33] both in terms of the yield and the ease of purification of the intermediates and final product. The nitro group was reduced to an amine with hydrogen over a Pd catalyst and reacted with thiophosgene to produce the benzylisothiocyanate derivative of DOTA. The reaction of the isothiocyanobenzyl-DOTA with the 5'-hexylamine linker arm of a synthetic primer (Fig. 4) covalently attaches the ligand to the oligomer [34].

Addition of the lanthanide ion to DOTA can be accomplished either before or after the ligand's attachment to the primer. We have not yet established which procedure gives the highest yield and fewest by-
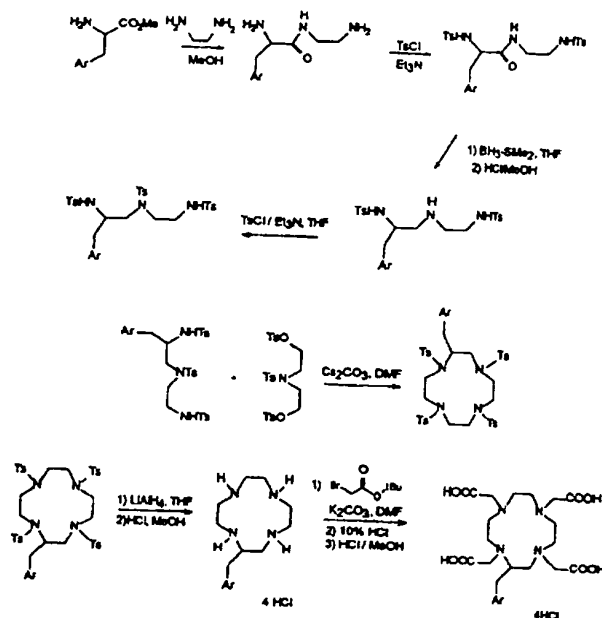
Figure 3. – General synthetic route to macrocyclic ring substituted tetraazacyclododecane tetraacetic acid (Ar-DOTA).
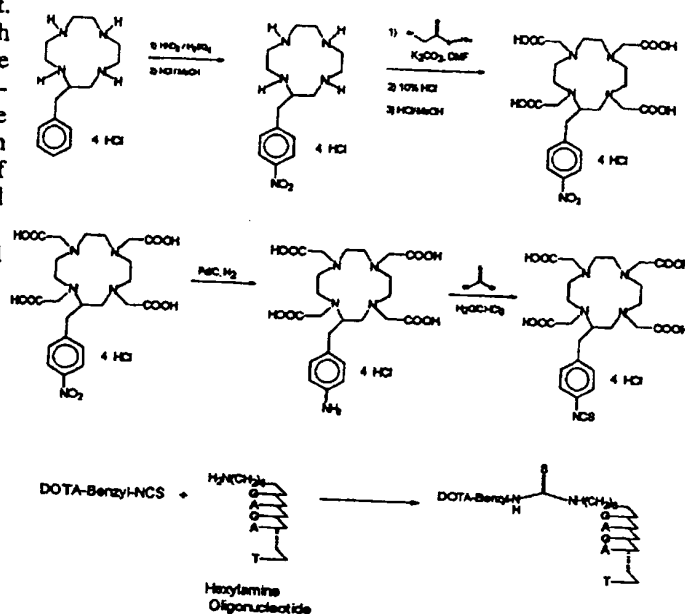
Figure 4. – Synthetic scheme incorporating a ring nitration step for the attachment of DOTA to oligonucleotides.

products, but we have taken the approach of adding the lanthanide ion last, thereby providing a greater versatility for sequencing applications. The HPLC-purified ligand-

primer complex has been reacted with an excess of aquated europium trichloride in a pH 7 buffer to generate the Eu labeled oligonucleotide. The Eu-oligonucleotide adduct was separated from excess Eu (III) by a final HPLC purification step [34].

Adding the isothiocyano-DOTA reagent to the hexyl-amine oligonucleotide while it is still attached to the controlled pore glass (CPG) column on which it was synthesized can eliminate several time-consuming HPLC purification steps. We have successfully employed this approach in the preparation of an Eu (III)-labeled 17-mer M13 sequencing primer. In the last automated step of hexylamine oligomer synthesis, the acid labile monomethoxytrityl (MMT) protecting group was removed from the 5'-terminal amine through the delivery of trichloroacetic acid (TCA) to the CPG column followed by the elution of the released MMT cation. The CPG column was removed from the machine and washed well with acetonitrile to remove any residual TCA. The isothiocyano-DOTA was then added to the CPG support-bound 17-mer and allowed to react overnight. The DNA-DOTA ligand adduct was then reacted with a large excess of EuCl3 for about one hour then washed well to remove uncomplexed Eu (III). At this point the Eu (III)-labeled oligonucleotide was cleaved from the CPG support with concentrated ammonium hydroxide. Purification by HPLC provided labeled primer suitable for use in sequencing reactions [34]. For sequencing applications requiring several lanthanide labels, the CPG-bound 17-mer oligomer could have been divided into separate portions for individual reactions with different Ln (III) salts and/or their isotopes.

Applications: pitfalls and promises

Numerous demonstrations of the use of ferrocene as a reporter for DNA have been performed. The successful coupling of the reporter to the priming oligomer was confirmed using routine diagnostics during its HPLC purification. UV absorption observations at 260 nm (nucleic acid peak) and 310 nm (ferrocene peak) were complemented by monitoring the electrochemical signature of the ferrocene with an on-line elecrolysis cell. The priming ability of the ferrocenecarboxylamide oligomer was demonstrated for DNA synthesis in both Sanger sequencing [16] and the PCR amplification of DNA [16,20]. These same DNA fragments have been subsequently subjected to separation, by size, in an electrophoretic field, then located and identified by RIS/MS [16,20], thus demonstrating both the stability of the DNA-ligand bond in this procedure and the ability of the detector to locate DNAs so labeled.

While some level of success has been realized for this approach to DNA fragment identification, the problem of iron contamination of many of the reagents and materials used routinely in molecular biology procedures has made the element unsuitable as a mass label [7,16]. For example, a nylon membrane, commonly used as a support for electrophoretically separated DNA fragments, was analyzed by RIS/MS and found to contain 8-10 ppm iron. This would seriously affect the ability of the instrument to detect a small amount of isotopic iron label in the high-iron-background signal. Although endogenous contamination poses a serious restraint to the use of iron as a mass label, ferrocene-based compounds can still serve as DNA reporters, although by virtue of their intrinsic electroactivity.

The intrinsic electroactivity of ferrocene provides an alternative means for identifying DNA fragments deriva-tized with this compound. Following their separation by size in an electrophoretic field, DNA fragments labeled with ferrocene are introduced into an electrochemical cell in which the working electrode has a fixed potential set somewhat in excess of the half-wave potential for oxidation of the electroactive reporter. In the case of ferrocenecarboxylic acid the half-wave potential is 620 mV vs SCE [35]. When the solution containing the ferrocene-labeled DNA enters the electrochemical cell, the anodic current resulting from the oxidation of the ferrocene moiety at the working electrode provides the means for detecting and identifying DNA fragments.

The half-wave potential of the Fe (II)/Fe (III) couple in ferrocene derivatives varies with the substituent conjugated to the cyclopentadienyl ring. Generally behaving in an additive manner, electron-withdrawing substituents added to the ring will increase the half-wave potential, whereas electron donating substituents will decrease this characteristic [36]. This makes possible a series of unique labels, in the form of different ferrocenecarboxylic acid derivatives (see Fig. 5). These derivatives can be synthesized quite easily using very well established and uncomplicated chemistry [37]. Efforts are currently underway to synthesize a number of ferrocene derivatives (in the form of NHS esters) for attachment to DNA primers.

Having determined iron's unsuitability as a mass label, the use of alternative elements for this purpose was explored. Various elements with relatively low background levels and numerous commercially available stable isotopes were found which could serve as possible DNA reporters. Tin, for example, had eight commercially available stable isotopes. RIS/MS analyses of the same nylon membrane analyzed for its iron content, showed only ppb levels of tin [16], making tin a much more likely candidate for mass labeling DNA.
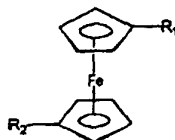
1) R_1=H, R_2=H. 2) R_1=COOH, R_2=H. 3) R_1=CH$_2$COOH, R_2=H.
4) R_1=C(O)(CH$_2$)$_2$COOH, R_2=H. 5) R_1=(CH$_2$)$_3$COOH, R_2=H.
6) R_1=(CH$_2$)$_3$COOH, R_2=C(O)NPh$_2$.
7) R_1=C(O)(CH$_2$)$_2$COOH, R_2=Br.
8) R_1=(CH$_2$)$_3$COOH, R_2=Br. 9). R_1=COOH, R_2=C(O)NPh$_2$.

Figure 5. - Some of the easily arrived at ring substitutions of ferrocenecarboxylic acids.

All eight commercially available stable isotopes of tin (^{112}Sn, ^{116}Sn, ^{117}Sn, ^{118}Sn, ^{119}Sn, ^{120}Sn, ^{122}Sn, ^{124}Sn) have been incorporated in TESPA carriers for use as DNA labels. Having a number of chemically identical, but individually distinguishable (by RIS/MS) reporters for DNA makes possible the multiplexing of DNA samples during their electrophoretic separation. With eight labels, eight different sequencing reactions can be co-separated in the same gel lane. Subsequent analysis of the gel by RIS/TOF-MS (resonance ionization spectroscopy/time-of-flight mass spectrometry), permits the simultaneous collection of sequence data for all eight DNAs, thereby greatly increasing the rate at which sequence data can be generated. With the use of a high-repetition-rate copper vapor laser and the eight tin reporters, one for each of eight DNAs, we estimate that approximately 2,000 individual DNA fragments can be analyzed in one second [16].

The usefulness of TESPA as a DNA reporter has been demonstrated in a number of practical applications. The label has been shown to be stable in the standard molecular biology procedures involved in Sanger dideoxy sequencing and PCR operations, and it readily survives the electrophoretic separation process [20, 26, 38, 39, 40]. Tin-labeled DNA fragments, arising from both PCR and Sanger sequencing reactions, have been successfully detected by RIS/MS in the gels in which they were electrophoretically separated [20, 26, 38, 39, 40].

With TESPA's usefulness as a mass label already demonstrated, it should be possible to extend this approach beyond the eight stable tin isotopes to the large number of lanthanide isotopes currently available. Preliminary experiments using Eu (III) complexed by isothiocyanobenzyl-DOTA as a DNA label have been quite promising. M13 Eu (DOTA) primers have been tested in the Sanger sequencing procedure against underivatized primers, and the reaction products compared following slab gel electrophoresis. To permit visualization of the products [^{32}P]-labeled deoxynucle-oside triphosphates were incorporated in the reactions. Subsequent autoradiographic results indicated that the presence of the label did not interfere with the enzymatic synthesis of DNA and that the label was stable to the conditions of electrophoresis. While we have not yet carried out the requisite RIS/MS experiments to establish sensitivity levels or otherwise show the utility of lanthanides as mass labels, we do not see any limitations nor do we anticipate any problems, in practice, that will inhibit their successful use in such an application.

Preliminary experiments have been done that concentrate on the luminescent properties of the Ln (III) ions as a means of identifying and locating electrophoretically separated DNA fragments. Results thus far have been inconclusive, which is understandable since the Eu (DOTA) label used did not contain a group such as naphthalene to serve as a detection enhancing antenna for luminescence spectroscopy. Efforts are underway to prepare a primer for M13 DNA which is labeled with an Eu (III) complex containing an antenna molecule to enhance the emission intensity.

The importance of demonstrating that energy transfer occurs efficiently from an organic triplet state to the Ln (III) ions in substituted DOTA ligands led to the preparation of Eu (III) complexed with 2-(2-naphthylmethyl)-DOTA. This derivative was selected since naphthalene has a triplet state which will appropriately transfer energy to the four Ln (III) ions of interest. Emission spectra were obtained by excitation of the naphthalene group as well as direct excitation of Eu (III). Sharp bands characteristic of the emission from f-f states of Eu (III) ions were observed. As expected the time dependence of the strongest emission band at 615 nm from Eu (NapDOTA) showed an increase at short times following pulse excitation with an exponential decrease at longer times. This rise in emission intensity is due to a rate limiting transfer of excitation energy from the naphthalene triplet to the emitting Eu (III) excited state. The emission data were fit to the reaction scheme

$$*Nap\text{-}Eu \rightarrow Nap\text{-}*Eu$$

$$Nap\text{-}*Eu \rightarrow Nap\text{-}Eu + hv$$
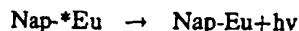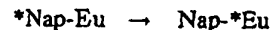
where * indicates the excited species. The fitted rate constant for intramolecular energy transfer was $1.2 \times 10^5\ s^{-1}$ and the derived lifetime of the emitting Eu (III) excited state was 0.18 msec. This proof-of-principle demonstration of energy transfer revealed the additional information that the rate of energy transfer from the naphthyl triplet state to the Eu (III) center is partially rate limiting in the emission from the complex [34]. Work is in progress to create an antenna molecule which is more intimately attached to the Eu (III) ion.

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Conclusions

By using metallic elements as labels for DNA, a variety of detection methods become available to analyze the products of sequencing reactions or other manipulations with DNA requiring the analysis of subfemtomole quantities. We have discussed labels that can be detected by RIS/MS (mass label), by emission spectroscopy, or by electrochemical reaction. Our interest in this area was prompted by the Human Genome Program. As advances are made in DNA sequence analysis, routine analysis of DNA will become commonplace and faster, less expensive methods of determining the sequence of small segments of DNA will be desired. We believe the unique analytical properties of metallic elements will make the chemistry we have developed become technologically valuable. We hope our efforts inspire

others to utilize metalloorganic compounds as labels for biological materials.

Acknowledgments

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6 A fully automated DNA sequencing apparatus is marketed by Applied Biosystems, Inc., Foster City, CA. It is based on the detection of fluorescent labels following separation of DNA fragments by slab-gel electrophoresis and is derived from development work in Hood's laboratory [3].

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